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SOME OBSERVATIONS ON INTERFERON PREPARED IN TISSUE CULTURES

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In 1957, Isaacs and Lindenmann reported that interferon was formed by the incubation of inactivated influenza virus with chick chorio-allantoic membranes; following this, a number of authors described a similar production of interferon in tissue cultures, using live virus (Burke and Isaacs, 1958; Henle, Henle, Deinhardt and Bergs, 1959; Ho and Enders, 1959; Tyrrell, 1959; Wagner, 1960). The specificity, as regards the cells from which the interferon is produced, has proved to be important. Thus, interferon prepared from calf kidney tissue cultures inoculated with live influenza virus was shown to possess a considerable degree of specificity, being active when assayed in calf kidney tissue cultures, but not active when assayed in chick chorio-allantoic membranes (Tyrrell, 1959). The following experiments were designed to show the presence or absence of such specificity in interferon prepared in a variety of tissue cultures. Certain other aspects of the action of interferon which might have a bearing on its possible use in man were also investigated.

METHODS

Tissue cultures

Rhesus and Cynomolgus monkey kidney cultures.—Primary monkey kidney cultures were obtained from the M.R.C. Biological Standard Control Laboratory, London. These were trypsinized and dispensed at a concentration of 60,000 cells/tube, using a modification of the method of Melnick (1955).

Calf kidney cultures.—Calf kidneys were obtained from the local abattoir and processed as described by Negroni and Tyrrell (1959). A cell concentration of about 60,000 cells/tube was used.

Human adult thyroid cultures.—Thyroids removed at operation (Royal South Hants Hospital, Southampton) were processed, using the method of Pulvertaft, Davies, Weiss and Wilkinson (1959).

Human embryo tissue cultures.—Human embryos were obtained and kidney and lung tissue cultures prepared as described by Tyrrell, Bynoe, Hitchcock, Pereira and Andrewes

(1960). Portions of skin and muscle were dissected from the embryos and tissue cultures prepared by a modification of the same method ; these cultures consisted almost entirely of fibroblasts.

Human amnion cultures.—Human placentae from normal deliveries and from caesarean sections were obtained from Odstock Hospital, Salisbury. The amnions were dissected clean and tissue cultures prepared by a method similar to that of Zitcer, Fogh and Dunnebacke (1955).

Tissue culture media

Tissue cultures were grown to confluence in media containing 5–20 per cent calf serum and 0·25 per cent lactalbumin hydrolysate in Hanks' balanced salt solution containing 0·035 per cent sodium bicarbonate and 100 units penicillin, 100 μg. streptomycin and 20 units mycostatin per ml. Confluent tissue cultures were maintained in 2 per cent inactivated horse serum and 0·25 per cent lactalbumin hydrolysate in Hanks' balanced salt solution with 0·105 per cent sodium bicarbonate and antibiotics added, or in medium 199 (Morgan, Morton and Parker, 1950) with 0·092 per cent sodium bicarbonate and antibiotics added.

Viruses

The strains used were :

Influenza A.—An allantoic fluid pool of the Kunz strain which had had a number of previous passages in embryonated eggs.

Parainfluenza 1.—An allantoic fluid pool of the Sendai strain of parainfluenza 1. This strain was well adapted to embryonated eggs.

ECHO 11.—A pool of the U strain of ECHO 11 (Philipson and Wesslén, 1958). This strain had previously had 21 serial passages in monkey kidney tissue cultures.

Coe.—A pool of virus with one previous passage in human amnion tissue cultures. This strain had been recovered from R.A.F. recruits by Dr. M. S. Pereira (Parsons, Bynoe, Pereira and Tyrrell, 1960).

Common cold viruses.—Two pools of the HGP strain of common cold virus (Tyrrell *et al.*, 1960). One of these had previously been passed 5 times in human embryo kidney tissue cultures ; the other pool had previously had 6 serial passages in human embryo kidney tissue cultures and a further 5 passages in monkey kidney tissue cultures. One pool of the FEB strain of common cold virus (Tyrrell *et al.*, 1960). This pool had previously had 8 serial passages in human embryo kidney tissue cultures.

Simian virus SV17.—Two pools of simian virus SV17 which had been derived from sick *Erythrocebus patas* monkeys (Goffe, unpublished). These pools had previously had one and four serial passages respectively in monkey kidney tissue cultures.

Preparation of interferon

Interferon was prepared by the inoculation of tissue cultures with the Kunz strain of Influenza A (about 10 haemagglutinating units to each tube culture and more, *pro rata*, when bottle cultures were inoculated). After 72 hr. incubation at 36°, the tissue culture fluids were harvested. Live virus was inactivated by dialysis at 4° against pH 2·1 0·1 M-citric acid-citrate buffer for 4 hr., followed by 3 changes of buffered saline. Tissue culture fluids from uninoculated tubes were dialysed in the same way and used as controls.

Assay of interferon

In each assay, 4 culture tubes were inoculated with 0·7 ml. interferon and 4 with control tissue culture fluid. Both control and interferon were allowed to act on the cells for 18–24 hr. at a final dilution of one in two. The tissue culture fluids were than changed and the cultures challenged with about 100TCD_{50} of the U strain of ECHO 11 virus, or, in the case of calf kidney cultures, a similar inoculum of the Sendai strain of parainfluenza 1 virus. The culture fluids were titrated by haemagglutination when the tissue cultures treated with control material had completely degenerated. Titrations were done at room temperature, using the method recommended by the World Health Organisation (1953). One per cent human erythrocytes were used in the case of ECHO 11 titrations and 0·5 per cent fowl erythrocytes in the case of parainfluenza 1 titrations. Dilutions were made in normal physiological saline buffered to pH 7·1 with 0·1 M-phosphate buffer. A 4-fold difference between

the mean titres of the control-treated and the interferon-treated culture fluids was considered a significant interference index (Hitchcock and Tyrrell, 1960) and is expressed in the tables as Log_2 units.

In some batches of interferon, a quantitative estimate was made of the amount of interferon present. This was done by assaying serial dilutions of interferon.

In some experiments, microplaques produced by the HGP strain of common cold virus (Tyrrell and Parsons, unpublished) were used as a basis for an assay. In these assays, the challenge dose of HGP virus used was about 10 microplaque forming units to each culture tube, and the interference index was assessed by comparing microplaque counts in interferon-treated with those in control-treated tubes. With this method, monkey kidney tissue cultures were used in all experiments and a 50 per cent reduction in microplaques was regarded as significant.

RESULTS

The cellular specificity of interferon prepared in different tissue cultures

Batches of interferon were prepared as described in a number of different types of tissue culture and these were assayed in homologous tissue cultures. Those found to produce significant interference when assayed in homologous cultures were then assayed in tissue cultures prepared from heterologous tissues. The methods used in the preparation and assay of the various batches were identical, the greatest variable being the tissue in which each batch had been prepared.

The results of these assays are given in Table I. They show a gradation existing in the cellular specificity of the interferons prepared in different tissues. Some, such as human amnion interferon, were only effective in homologous tissue cultures. Others, such as calf kidney and human adult thyroid interferon, were active in some tissues but not in all. Interferon prepared in rhesus monkey kidney tissue cultures was unique in that it was active in every type of tissue in which it was tested.

TABLE I.—*The Cellular Specificity of Interferon Prepared in Different Tissue Cultures*

Interferon assayed in tissue cultures of :	Results of assays of batches of interferon prepared in :			
	Human adult thyroid	Rhesus monkey kidney	Calf kidney	Human amnion
Human adult thyroid	4/5*	1/1	1/2	0/1
Rhesus monkey kidney	0/2	5/5	0/3	0/1
Calf kidney	2/4	2/2	4/5	0/1
Human amnion	0/1	1/1	1/1	1/1
Human embryo kidney	1/2	2/2	1/2	NT
Human embryo lung	1/3	1/1	1/3	0/1
Human fibroblast	NT	1/1	NT	NT
Cynomolgus monkey kidney	0/2	2/2	0/1	NT

* Numerator : number of assays where there was significant protection.
Denominator : total number of batches of interferon assayed.
NT = Not tested.

Not all batches of interferon were active when assayed in homologous tissue cultures. One out of 5 batches of human adult thyroid interferon and one out of five batches of calf kidney interferon were inactive when tested in this way : they were not subsequently assayed in heterologous tissue cultures.

The protection afforded by interferon against challenge by respiratory viruses

It is clear from the preceding section that certain interferons could protect tissue cultures against parainfluenza 1 and ECHO 11 virus infection. The range of challenge viruses was therefore extended to include other respiratory viruses.

Tissue cultures treated with rhesus monkey kidney interferon were challenged with the HGP and FEB strains of common cold virus, with Coe virus and with SV17 (a virus known to be associated with respiratory illness in *Erythrocebus patas* monkeys). Significant protection was observed against all these viruses except SV17. Interferon prepared in rhesus monkey kidney tissue cultures was capable, therefore, of protecting against infection by respiratory viruses of various kinds. These included a myxovirus (parainfluenza 1) an enterovirus (ECHO 11) and 3 of the newer respiratory viruses (HGP, REB and Coe). SV17, a simian virus resembling an adenovirus, was not susceptible. In the experiment summarized in Table IV, another difference in susceptibility was observed, the growth of HGP virus being apparently inhibited by lower concentrations of interferon than was that of ECHO 11.

Some time-relations of the action of interferon in tissue culture

The duration of protection given by one overnight treatment with interferon was investigated. Calf kidney and rhesus monkey kidney tissue cultures were treated with homologous interferon or with control fluid and were challenged with parainfluenza 1 or ECHO 11 viruses on varying days after treatment. The results of these experiments are summarized in the upper part of Table II. Other groups of cultures were treated on days 1–4 with quarter doses of interferon or control fluid and all the groups were challenged on day 5. The results of these experiments suggest that one overnight treatment protected for up to 5 days and also that treatment given in divided doses over 4 days was as efficacious as an undivided dose on day one.

TABLE II.—*The Duration of Protection Given by Overnight Treatment with Interferon*

Day (or days) of treatment with interferon	Day of challenge	Interference index* of interferon prepared in : Rhesus monkey kidney tissue cultures (assayed in similar cultures, using ECHO 11 as challenge virus)	Interference index* of interferon prepared in : Calf kidney tissue cultures (assayed in similar cultures using parainfluenza 1 as challenge virus)
1	2	3·6	3·0
1	4	2·4	4·8
1, 2, 3, 4†	5	3·3	3·4
1	5	3·9	3·0
1	8	2·3	−0·4

* For each interference index, we have compared the titre of one group of cultures which was inoculated with interferon with another similar group which was inoculated with control fluid at the same time.

† This group of cultures was treated on days 1, 2, 3, 4 with quarter doses of interferon.

The duration of treatment necessary to give protection was also investigated. Calf kidney tissue cultures were treated with homologous interferon for 1 hr., 6 hr.

and overnight and then the interferon was removed from the culture tubes and fresh medium substituted. The cultures were then challenged with parainfluenza 1 virus, either immediately after changing the medium or after a further 18–24 hr. The results of these experiments are given in Table III. In neither group was there any significant protection in those cultures treated for only one or 6 hr., a result in agreement with that of Burke and Isaacs (1958).

TABLE III.—*The Duration of Treatment with Interferon that is Necessary to Give Protection*

Duration of treatment with interferon	Interference index in cultures challenged: Immediately after end of treatment with interferon	Interference index in cultures challenged: 18–24 hours after end of treatment with interferon
1 hr.	0·0	0·5
6 hr.	1·1	1·5
Overnight	2·9	2·5

The stability of interferon prepared in tissue cultures

Interferon prepared in a serum-containing medium was active and stable, but the presence of serum made it unsuitable for administration to man or for concentration by freeze-drying, etc.

Interferon was therefore prepared in tissue cultures maintained in a serum-free medium (medium 199) and its stability was investigated. In different experiments, the interfering activity of interferon prepared in calf kidney and in monkey kidney tissue cultures was found to be abolished by 3–6 cycles of freezing (−20°), and slow thawing at room temperature. Because of this instability, preservation or concentration by freeze-drying was not possible, as all interfering activity was lost during this process.

Possible preservatives against this loss of activity were investigated, using interferon prepared in calf kidney and in rhesus monkey kidney tissue cultures. It was found that the addition of 1 per cent starch, 1 per cent dextran, or 1 per cent glycerol gave no protection against repeated (3–6 times) freezing (−20°) and thawing at room temperature. The addition of 1 per cent gelatin, however, conferred complete protection on the interferon against 6 similar cycles of freezing and thawing.

Interferon was then prepared in rhesus monkey kidney tissue cultures and gelatin added to give a final concentration of 0·1 per cent. This material was dialysed against distilled water for 18 hr. at 4° and freeze-dried *in vacuo*. When reconstituted with distilled water to one tenth of its original volume, it was found that there had been a corresponding 10-fold increase in titre over the unconcentrated interferon. The results of this experiment are shown in Table IV.

DISCUSSION

Consideration of the possible use of interferon in man as a preventive agent against viral infections immediately raises a number of questions. One of these is the nature of the cell system in which to prepare interferon for use in man. Isaacs and Lindenmann (1957) showed that interferon prepared in chorio-allantoic

TABLE IV.—*Assay of Interferon After Concentration by Freeze Drying*

Challenge virus used	Virus growth detected as :	Dilution of interferon				Control	Titre of Interferon
		Undil.	4	16	64		
HGP *	Total number of plaques in four tubes	1	1	16	18	57	>64
	Per cent of control	*1·7*	*1·7*	*28*	*32*		
ECHO 11*	Mean HA units/tube	10	14	18	59	96	16
	Per cent of control	*10·4*	*15*	*19*	*61*		
HGP†	Total number of plaques in four tubes	1	1	4	8	57	>64
	Per cent of control	*1·7*	*1·7*	*6·8*	*14*		
ECHO 11†	Mean HA units/tube	13	20	21	78	92	16
	Per cent of control	*14*	*21*	*23*	*84*		

* Unconcentrated interferon used.
† Interferon used concentrated 10 × and reconstituted to original volume.

membranes was active when assayed in the same system. But when interferon prepared in chorio-allantoic membranes was assayed in calf kidney tissue cultures, no interference was observed (Tyrrell, 1959). A similar failure to protect heterologous tissues was described by Wagner (1960) and by Isaacs and Westwood (1959). The present investigation extends these observations and suggests that interferon assayed in a variety of tissue cultures may be active or inactive, according to the cellular specificity of the tissue cultures in which the interferon has been prepared. For example, interferon prepared in rhesus monkey kidney tissue cultures, unlike interferon from any other source, was active in 5 types of human tissue culture. It was also active in calf kidney tissue cultures, an unexpected finding which deserves further investigation. It is also possible that some of the differences in specificity observed appeared because cultures of some tissues produced more interferon than others.

From this, although extrapolation from individual tissues to the complete organism is, of course, hazardous, it appears that rhesus monkey kidney tissue cultures may be the ones of choice for the preparation of interferon to be used in man. Should this extrapolation from human tissue cultures to man himself be justified, the present work also suggests that interferon prepared in rhesus monkey kidney tissue cultures offers hope of protecting man against a variety of respiratory viruses, possibly for periods of up to one week. Concentration of interferon by freeze-drying may make it practicable to administer to man over conveniently short periods doses which are large enough to be effective.

The mode of action of interferon, however, is at present imperfectly understood, as are any possible noxious effects, immediate or remote. The need for careful safety tests before its use in man is attempted becomes therefore self-evident.

SUMMARY

Interferon was prepared in rhesus monkey kidney, calf kidney, human adult thyroid and in human amnion tissue cultures by the inoculation of live influenza A virus. When assayed in heterologous tissue cultures, the presence of cellular specificities of different degree became evident. Interferon prepared in rhesus

monkey kidney tissue cultures was active in all cultures in which it was assayed, unlike that prepared in human amnion cultures, which was only active when assayed in homologous tissue cultures. Interferon prepared in calf kidney and in human adult thyroid tissue cultures occupied an intermediate position.

Interferon prepared in rhesus monkey kidney tissue cultures protected cultures against challenge by the HGP and FEB strains of common cold virus, by Coe virus, by ECHO 11 and parainfluenza 1 viruses, but not by SV17. Overnight treatment with interferon was necessary to provide protection and this protection against challenge lasted for 5–8 days. Using gelatin as a preservative, it was possible to concentrate monkey kidney interferon 10-fold by freeze-drying, with a corresponding increase in titre.

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OBSERVATIONS ON THE NATURE OF THE ENLARGEMENT, THE REGENERATION OF THE NERVES, AND THE FUNCTION OF THE CANINE RENAL AUTOGRAFT*

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CANINE renal autografts were observed for periods up to 12 months to determine : the nature of the enlargement of the graft, a usual sequela when the autograft is an only kidney ; the time of appearance and time required for the regeneration of nerves ; function of the autograft, particularly as it might be related to enlargement of the graft and the regeneration of its nerves. Quantitation of the factors in enlargement of the graft included an initial weighing and linear measuring, and needle biopsy. Neural regeneration was studied by microscopic examination of the nerves on the graft side of the anastomoses after staining of the tissues by the Bodian (1936) method. Function of the renal graft was tested by the response to water-loading and water-deprivation, urine analysis, determination of phenolsulphonphthalein excretion, blood urea nitrogen levels and the arterial blood pressure. From previous experience (Bricker, Straffon, Mahoney and Merrill, 1958 ; Murray, Lang, Miller and Dammin, 1956), it was expected that no significant compromise of renal function would occur.

MATERIALS AND METHODS

Randomly selected healthy female mongrel dogs weighing 12–16 kg. were used and were divided into the following 3 groups.

Group 1 (*Delayed autografting of the solitary kidney*).—Three animals comprised this group. The initial operation was nephrectomy, and 3 months later the remaining kidney was transplanted to the iliac vessels.

Group 2 (*Simultaneous nephrectomy and autograft*).—Four animals. At the initial operation, unilateral nephrectomy was performed, and the remaining kidney was autografted.

Group 3 (*Nephrectomy and pedicle clamping*).—Four animals. At the first operation one kidney was removed ; the pedicle of the remaining kidney was clamped for 1 hr.

Operative techniques

Autotransplantation to the iliac fossa (Murray *et al.*, 1956). After anaesthetizing the animal with intravenous sodium pentobarbital (30 mg./kg.), a midline abdominal incision was made, extending from xiphoid to pubis. The common iliac artery and vein were dissected for a distance of about 3 cm. from the aortic and inferior vena caval bifurcations. After clearing all the areolar adventitial tissue from these vessels, bulldog clamps were placed at their origins and the vessels were divided slightly proximal to the internal iliac origins.

The contralateral kidney was dissected after an incision had been made in the peritoneal reflection leaving an envelope of peritoneum on the anterior two thirds of the renal surface. The renal vessels were then isolated, cleared of areolar tissue and nerves, and ligated and

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divided as closely as possible to their origins. The ureter was transected approximately 5 cm. from the renal hilum and the distal segment was ligated.

The kidney was weighed measured in its longitudinal and lateral axes and biopsied by piercing the cortex with a hollow needle (internal diameter : 3 mm.) using a rotatory motion, thereby obtaining a cylindrical plug. Bleeding from such biopsy sites was usually arrested by a single stitch.

The kidney was then put into the iliac fossa and the anastomosis begun. The arterial anastomosis was achieved by the continuous, everting method of Carrel (1908, 1911) ; the venous anastomosis was executed with a continuous. " over-and-over " stitch. The suture material was 5–0 vascular silk on an atraumatic needle. The ureterovesical anastomosis was completed by embedding the " fish-mouthed " end of the ureter in the bladder lumen and securing it with 2 anchoring stitches. The kidney was fixed to the tissues lateral to the psoas muscle by means of stitches passed through the renal capsule and parietal peritoneum, and the midline abdominal wound was closed with chromic catgut and nylon retention sutures. The latter were removed on the 14th post-operative day.

During the procedure each animal received 300 ml. of 5 per cent glucose in water intravenously. The duration of total renal ischaemia never exceeded 1 hr.

Renal pedicle clamping.—The animal was anaesthetized and the abdominal cavity entered as in the autografting procedure. The renal vessels were isolated without stripping their nerves or areolar tissue. Bulldog clamps were applied to the artery vein and ureter for 1 hr. When no bleeding occurred after lightly pricking the organ's surface, it was assumed that complete ischaemia obtained. During this period of interrupted blood flow the opposite kidney was removed. A tissue specimen was taken from both kidneys, using the hollow needle. After the ischaemic period was completed, the abdomen was closed in the manner described above.

All animals received 300 ml. of 5 per cent glucose in water during the procedure.

No anticoagulants were used in either the autografting or pedicle clamping operations.

Measurement and biopsy

At monthly intervals concurrent with the tests of function, each animal was subjected to laparotomy. In order, the kidney was measured in its sagittal and transverse direction, and a hollow needle biopsy plug was obtained.

Studies of function

For 3 days prior to each operation each dog had a daily urine analysis, intake and output recording of fluid volume, and testing of phenolsulphonphthalein (PSP) excretion. For the latter, each animal was given a 300 ml. water load (by gastric tube), and PSP (1 ml.) was given intravenously 15–20 min. later. Aliquots of urine were obtained at 30, 60 and 90 min. after PSP injection. For testing response to water restriction, each animal was restricted to a fluid intake of 300 ml. during one of the three 24-hr. test periods.

Urine collection was by means of a No. 12 (French) Foley indwelling catheter, connected to a flexible plastic bag.

Blood urea nitrogen was determined in all dogs at the outset of the experiment, and at 1–2-monthly intervals thereafter. Immediately before each operation, prior to anaesthesia, arterial blood pressure was measured in each animal by means of direct femoral arterial puncture with a No. 18 needle connected to a mercury manometer.

Termination of the study

At the conclusion of each experiment, the animal was killed by nembutal overdosage, and the kidney was immediately removed. After the kidney was weighed and measured (before draining its contained blood), specimens of the hilar structures (in cross and sagittal section) and the renal parenchyma were obtained.

Histological studies

One half of each renal tissue specimen was immediately frozen in air, using ether-dry ice as the coolant ; the remaining half was fixed in 10 per cent formol-saline. The frozen tissues were sectioned, and stained with haematoxylin and eosin. The fixed tissues were embedded in paraffin, sectioned, and stained with haematoxylin and eosin and periodic acid-Schiff stain.

Using an American Optical Company micrometer, all of the glomeruli of each frozen specimen presenting a midsagittal plane were measured. That is to say, the entry point of the preglomerular arteriole and the exit point of the convoluted tubule were both visualized in the same glomerulus.

The remainder of the parenchyma was also examined, in the fixed specimens, with especial attention to inflammatory response, fibrosis, tubular configuration, and mitosis.

The renal hilar structures were fixed in 10 per cent formol-saline, and stained by the method of Bodian (1936). These were examined to determine the appearance of neural elements, vascular structures, and the ureter.

RESULTS

In the Table are summarized the data relating to hypertrophy (by weight) of the kidneys of groups 1 and 2. Two of the three kidneys of group 1 (wherein grafting was performed after the kidney had been in the solitary state for 3 months) demonstrated insignificant weight changes after grafting. The third kidney of group 1 was pyelonephritic. In contrast, the kidneys of group 2 (simultaneous nephrectomy and autograft) underwent hypertrophy of 21–55 per cent during the 9–12 month interval.

TABLE—*Comparison of Changes in Weight of Kidneys which were Autografted after Functioning as a Solitary Kidney (Group I) with Kidneys Autografted at the Time of Nephrectomy (Group II)*

Animal No.	Weight of kidney at time of autograft (g.)	Duration of graft (months)	Weight after autograft (g.)	Absolute change	Per cent change
Group 1					
22	61·9	11	96·7*	34·8	56·0
31	50·3	12	49·0	−1·3	−2·6
32	52·9	12	60·8	7·9	14·9
Group 2					
23	46·3	12	58·5	12·2	20·9
25	31·0	11	48·2	17·2	55·5
26	44·0	9	57·8	13·8	31·4
35	27·0	12	35·5	8·5	31·5

* This kidney developed pyelonephritis, which accounted for most, if not all, weight gain.

Studies of gross function

There were no significant or persistent changes in arterial blood pressure, blood urea nitrogen, phenolsulphonphthalein excretion, and the response to water loading and water deprivation. This applied in all groups, with the exception of the one animal in group 1 with severe pyelonephritis. In this case, the blood urea nitrogen rose to 125 mg./100 ml. shortly before death in the eleventh post-operative month.

Histological findings

An average of 4 glomeruli cut in the midsagittal plane were found in each tissue section. In all 3 groups of animals, the control and test glomerular diameters were remarkably constant, and in the 0·13–0·15 mm. range. Therefore, there was no detectable glomerular enlargement in any group.

Aberrations from normal histological patterns were inconstant and minimal. An occasional hyaline or epithelial cast and occasional focal pyelonephritic scars were found. In Figs. 1 and 2 are depicted photomicrographs of renal parenchyma before and after autografting.

Evidence for nerve regeneration was established by the presence of non-fragmented axis cylinders within nerve bundles cut in a longitudinal plane. Because of the thread-like character and circuitous route of the renal nerves travelling in the loose adventitia surrounding the hilar vessels, obtaining nerves cut in longitudinal section required several histological sections of each specimen. In some slides several nerve bundles could be found travelling short distances parallel to the plane of sectioning. It was in such segments that the condition of the axis cylinders was assessed.

From another experiment, it was learned that one week following autografting, the nerve fibre architecture of the hilar structures was completely destroyed and there was extensive dissolution and necrosis of Schwann cells and only a rare axis cylinder fragment could be identified (Fig. 3).

Three months after autografting, the ratio of axis cylinders to Schwann cells was reduced, due mainly to proliferation of the Schwann cell component. These cells were oriented parallel to the long axis of the nerves. Between many Schwann cells, non-fragmented axis cylinders were seen (Fig. 4). Because of the thickness of the tissue sections required for optimal silver impregnation (20 μ) these nerve cylinders could be more easily followed and appreciated under the microsope than in a photomicrograph of a single optical plane.

At 6 months after autografting, it was especially appreciated that nerve regeneration is a variable process. In some of these specimens there were significant degrees of fibrosis and chronic inflammation within the perivascular fibro-fatty tissue possibly related to surgical trauma and haematoma formation. Possibly purely local mechanical factors accounted for lack of regeneration. In other regions, however, there was evidence of nerve regeneration and a relatively normal nerve pattern was present (Figs. 5 and 6).

DISCUSSION

Innervation of the kidney

The importance of renal nerves in controlling renal function has long been in dispute. Since Holbrook's early anatomical study (1883), several investigators have elucidated the nerve elements found throughout the kidney and its related structures (Gruber, 1933 ; Mitchell, 1951).

The genito-urinary system is innervated by fibres from spinal cord segments T. 4 to L. 5. The renal plexus itself is fed by the semilunar ganglion, which communicates with the greater and lesser splanchnic nerves, and with the vagus nerves. The majority of these neurons derive from the sympathetic system, which provides both vasoconstrictor and vasodilator effects. It is well established that stimulation of the peripheral end of the divided splanchnic nerve results in renal contraction, presumably due to vasoconstriction. However, Bradford (1889) in an early study in dogs wherein the spinal roots were stimulated, found that the effect on renal vasculature depended on the rate of stimulation : quick rates (10–50 per sec) produced vasoconstriction, and slow rates (1 per 1–2 sec) produced vasodilatation.

It has been concluded that the majority of renal nerves are postganglionic, since very few ganglion cells have been found in the hilus or parenchyma. Free nerve endings have been identified in the adventitia of the small blood vessels, the glomeruli, and in the tubules (Harman and Davies, 1948 ; Mitchell, 1951).

The ureters are innervated from the renal, spermatic (or ovarian), and hypogastric plexuses. These nerves follow the small arteries of the ureteral adventitia (" Grund-plexus " of Engelmann), distributing many fibres to the tunica muscularis and a few fibres to the mucosa. Both medullated and non-medullated fibres have been found. A few ganglion cells appear throughout the entire length of the ureter, but the majority occur in the lowermost 3–4 cm. Ureteral peristalsis, however, is known to be primarily dependent on intrinsic activity of the smooth muscle ; it can function quite independently of nerve supply.

Quinby (1916) was one of several earlier workers who demonstrated that total renal denervation, by renal autografting, does not significantly impair function. Numerous, more recent investigations purport to demonstrate that varying degrees of increased water and sodium excretion result from renal denervation by other methods, including excision of the splanchnic nerves, stripping of the renal vessels, and phenolization of the renal pedicle (Bricker *et al.*, 1958 ; Meilman and Winer, 1953 ; Page, Baxter, Reem, Scott-Baker and Smith, 1954 ; Surtshin and Schmandt 1956). Many of these studies show such wide variance in both control and test groups that interpretation is made difficult.

It is, of course, a certainty that nerve degeneration occurs after division of the renal pedicle, but no data are yet available defining the time of appearance of regeneration. Nor has the effect of moving the pedicle to the iliac vessels been evaluated, a situation in which cells responsible for regeneration may ultimately innervate a structure new to this site.

The presence of axis cylinders in portions of the renal nerves distal to the line of anastomosis suggests to us that a certain amount of neural regeneration had taken place as early as 3 months. Re-proliferation and orderly longitudinal alignment of Schwann cells in itself implies a favourable environment for axis

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EXPLANATION OF PLATE

FIG. 1.—Needle biopsy specimen of kidney at the time of autografting. (Animal No. 35.) (H. and E.) ×64.

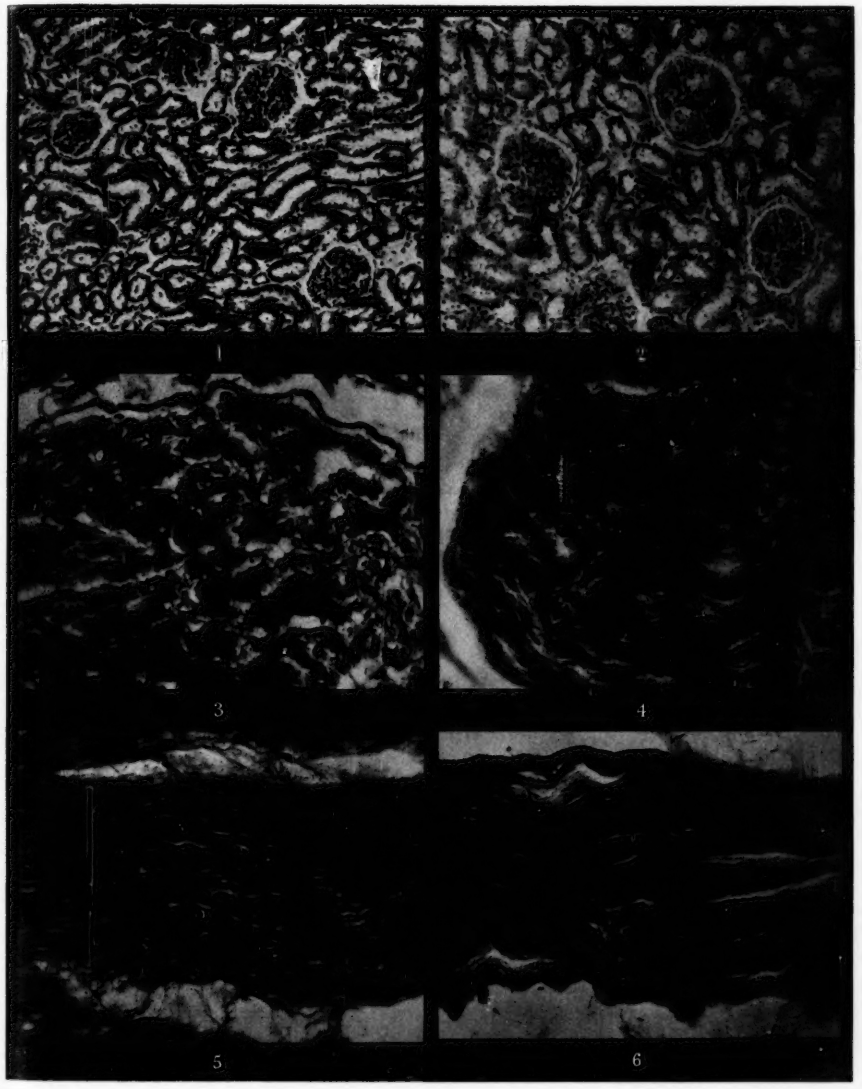
FIG. 2.—Histological appearance of the kidney shown in Fig. 1, 12 months following autografting. No obvious morphological change provides an explanation for the increase in weight. Although the interstitial tissue is more prominent and the tabular epithelium is slightly taller it is difficult to visualize how these account for the 31·5 per cent increase in weight. (Animal No. 35.) (H. and E.) ×64.

FIG. 3.—Sympathetic nerve distal to the line of vascular anastomosis one week following autografting. There is dissolution of nerve fibre architecture, fragmentation of axis cylinders and pyknosis of Schwann cell nuclei. (Bodian.) ×203.

FIG. 4.—Sympathetic nerve distal to the line of vascular anastomosis 3 months following autografting. There is early reorganization of nerve fibre architecture. Schwann cells are numerous and are oriented in parallel bundles. Axis cylinders are visible in some regions as thin filaments between Schwann cells. (Bodian.) ×203.

FIG. 5.—Sympathetic nerve distal to the line of vascular anastomosis 6 months after autografting. Neural architecture has been virtually reconstituted. Numerous wavy axis cylinders are present. Schwann cells are not as prominent as at 3 months but are more prominent than in a normal nerve. (Bodian.) ×203.

FIG. 6.—Sympathetic nerve lying in loose adventitia of the renal pedicle of a normal non-grafted canine kidney. (Bodian.) ×203.



Couch, McBride, Dammin and Murray.

cylinder regrowth. These features are in marked contrast to the findings at one week, at which time complete necrosis of nerve bundles was present. Adverse local factors such as chronic inflammation and fibrosis possibly related to surgery and haematoma formation most probably account for the absence of axis cylinder regeneration in some of the 6-month specimens.

In an earlier study (Murray *et al.*, 1956), wherein only haematoxylin-eosin stained sections were used for the identification of nerves, regeneration was not recognized.

In a series of experiments in cats, Lee (1921) found that partially excised cervical sympathetic trunks regenerated fairly completely over an average interval of 35 days. The shortest interval was 26 days. Stimulation techniques as well as anatomical methods were used for estimating the degree of regeneration.

A better parameter for assessment of renal nerve regeneration might consist of studies designed to demonstrate functional variations in renal excretory mechanisms in the autografted specimen following stimulation of specific nerves.

Ischaemia

When its duration does not exceed 2 hr., occlusion of the renal artery has been found, in dogs, not to result in serious functional impairment (Hamilton, Phillips and Hiller, 1948). The animals of group 3 were used as controls pertinent to this factor. No differences, either functional or anatomical, were found to exist between these kidneys and the autografts. It is concluded that the denervation incidental to autografting is not followed by detectable histological change of the renal parenchyma.

Hypertrophy

Enlargement of the solitary kidney is a well known process occurring secondary to interruption of function in the contralateral kidney, whether by agenesis, resection, or ureteral ligation. The specific agents responsible have not been identified, but there is substantial evidence that they are humoral (Berglund, Medes, Huber, Longcope and Richards, 1935). It has been estimated that renal hypertrophy can occur in 20–30 days in man (Berglund *et al.*, 1935).

In the dog, compensatory hypertrophy is chiefly ascribable to enlargement (both lengthening and thickening) of the convoluted tubules; although some investigators have described a slight increase of interstitial tissue. In the rat, the number of glomeruli does not increase (Arataki, 1926).

Hypertrophy would be measured more accurately by a volumetric technique which did not require excision of the organ. In this manner, sequential changes of size could be determined in any given kidney during any desired time interval. But, because the kidney is an irregularly shaped structure, and only 2 axes can be measured without mobilizing the organ, and because of probable variations in degree of renal vasoconstriction, our attempts at linear measurement yielded inconclusive data.

On the basis of gain in weight, the kidneys of group 2 (wherein autografting was performed simultaneously with nephrectomy of the opposite kidney) increased in size by 21–55 per cent somewhere before the end of the 9–12 month interval. In group 1 (wherein autografting was performed 3 months after removal of the opposite kidney), one of the grafted kidneys unfortunately developed pyelo-

nephritis. The remaining 2 kidneys showed slight changes in weight, which were well within the probable limits of error in the method.

These data indicate that, first, compensatory hypertrophy occurs after autografting if the contralateral kidney is removed at the time of the autograft, and, second, no significant further increase in size occurs if a kidney, previously solitary for 3 months, is autografted. It is justifiable to assume that hypertrophy had occurred in the latter type of kidney during the 3 month period prior to transplantation.

Additional parameters

The transplantation manoeuvre did not alter the mean arterial blood pressure. As other investigators have shown, the blood urea nitrogen, the rate of PSP excretion, and the responses to water loading and water deprivation were also not significantly affected. These functional studies were not intended as a precise physiological inquiry ; they were performed to provide a simple means of determining gross functional status of each kidney at any given time.

SUMMARY

Over periods of 9–12 months, 7 canine renal (iliac) autografts were studied in detail. Four additional animals were followed after renal-pedicle clamping for 1 hr., without denervation.

Anatomical evidence of nerve regeneration appeared in the five autografted specimens which were examined. Regeneration was found at 3 months, the first interval at which this parameter was studied. It appeared to be complete by 6 months.

When there was simultaneous nephrectomy and autografting, hypertrophy was observed, but, when autografting was delayed for 3 months after nephrectomy, no further increase in renal size occurred.

Sequential biopsies of renal parenchyma, obtained at monthly intervals, revealed no increase of glomerular diameter at any time. Moreover, there were no consistent changes in histological appearance of the parenchyma, with the exception of one dog in group 1, in which pyelonephritis developed.

This study, like others described, establishes the observation that the autograft as an only kidney is life-sustaining, shows no constant histological abnormalities, and has a normal function.

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SUCCINIC DEHYDROGENASE OF WEANLING NORMAL AND OSTEOLATHYRITIC RATS

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OSTEOLATHYRISM, a condition induced experimentally by the administration of β-aminopropionitrile and related compounds to laboratory animals, notably rats, has been widely studied. The nature of the pathological changes observed has been extensively investigated (Selye, 1957) but little biochemical correlation has been attempted. It has been suggested, in view of the chemical constitution of the toxins involved, that the primary change may be an impairment of carbohydrate metabolism (Levy, 1959); and Ramamurti and Taylor (1959) have suggested, on theoretical grounds, that a block of aerobic glycolysis might explain the observed defects in epiphyseal cartilage in lathyritic animals.

Recent studies have shown that an oxidative metabolism exists in epiphyseal cartilage; and according to Castellani and Zambotti (1954) and Whitehead and Weidmann (1959) succinic dehydrogenase is localized to the region of hypertrophic cells, but Hiatt and Shorr (1953) and Follis and Melanotte (1956) favour a more widespread distribution.

In the experiments described in this paper, the succinic dehydrogenase activity of liver and costal cartilage of normal and lathyritic rats has been studied histochemically and the activity of this enzyme in liver has been quantitatively measured using a spectrophotometric method.

MATERIALS AND METHODS

Three litters of freshly weaned Wistar rats (35–45 g.) were divided into 2 groups irrespective of sex. One group was fed on powdered rat diet No. 41 (Rank), and the other on the same diet to which semicarbazide hydrochloride had been added to a final concentration of 0·3 per cent (w/w). Both diets were moistened slightly and formed into rough pellets. From each litter 1 normal and 1 lathyritic animal were examined histochemically but the liver activity of all animals was measured. It was found that the control group gained 15–20 g. per week whereas the experimental group gained only 0–5 g. per week. Accordingly, in 1 litter of 3 animals, 1 was fed *ad libitum*, whilst another was given a reduced amount of the same diet so that its weight gain was comparable with that of the animal on the semicarbazide hydrochloride diet. Each animal of this litter was studied histochemically.

Animals were killed after 14–21 days, at which time all the animals on the lathyritic diet showed the pathological changes normally associated with osteolathyrism. The costochondral region of the rib was excised immediately after death and examined within a few min. The whole liver was removed and treated immediately or stored at a temperature of about $-20°$ prior to subsequent experimentation.

Histological demonstration of succinic dehydrogenase activity.—Frozen sections (8–15 μ thick) of unfixed tissue were cut on a " Pearse " cold microtome and succinic dehydrogenase activity demonstrated using the Nitro-BT method of Nachlas, Tsou, de Souza, Cheng and Seligman (1957), except that in the case of the cartilage it was found advisable to increase the final concentration of Nitro-BT in the incubation mixture to 1 mg./ml. and to incubate the sections at 37° for 2–3 hr.

Chemical determination of succinic dehydrogenase activity.—The morphological alterations of epiphyseal cartilage specifically related to lathyrism are restricted to the narrow zone in the region of hypertrophic and proliferating cells ; in view of the weak reaction for succinic dehydrogenase in cartilage as shown histochemically and the difficulties in obtaining samples of altered epiphyseal cartilage free of neighbouring marrow or muscle which have high activity, chemical assay of succinic dehydrogenase of epiphyseal cartilage was considered to be impracticable.

Weighed portions of liver were homogenized in ice-cold 0·1 M phosphate buffer, pH 7·2 (4–5 per cent w/v) and assayed in triplicate by the method of Slater and Bonner (Bonner, 1955) modified as follows :—The enzyme reaction was carried out in a conical centrifuge tube at 37° and terminated, after exactly 5 min., by the addition of trichloracetic acid to a final concentration of 5 per cent (w/v). The suspension was then centrifuged and the optical density read at 400 mμ. Trichloracetic acid and centrifugation has the advantage of providing a stable colour. Control estimations in the absence of homogenate and in the absence of succinate were also performed. Duplicate portions of the liver of each animal were assayed.

The activity is in arbitrary units of decrease in optical density/minute. Results are expressed in 3 ways ; on a liver wet weight and mg. nitrogen basis and according to the following equation :—

$$\text{Activity/100 g. body weight} = \frac{\text{Activity/g. wet weight} \times \text{Liver weight} \times 100}{\text{Total body weight}}$$

Other procedures.—Routine procedures were used for preliminary histological examination and nitrogen was determined by a micro-Kjeldahl method.

RESULTS

Histological

Rib

In approaching the rib-cartilage bone border from the cartilage side, 4 zones may be recognized :—1. A zone of evenly dispersed cartilage cells (the resting zone). 2. A proliferative zone in which the cells are gathered in groups and columns. 3. A hypertrophic zone in which the individual cells in the columns are much enlarged. 4. A single or double cell layer in which the hypertrophic cells are enclosed in mineralized cartilage matrix.

Normal : unrestricted diet (*Fig.* 1).—Enzyme activity was detected in the cytoplasm of all chondrocytes in the form of sharply defined blue granules, closely packed in the smaller cells such as those in the perichondrial region but dispersed in the larger cells. The staining reaction was similar in the resting and proliferative zones but, even allowing for the differences in cell size, the reaction appeared less intense in the hypertrophic cells, particularly in the mineralized hypertrophic zone.

Normal : restricted diet (*Fig.* 2).—The diminished growth rate of these animals was reflected in a considerable reduction in the depth of the proliferative and hypertrophic zones. Nevertheless the overall pattern and intensity of enzyme activity was essentially the same as in the group of animals on an unrestricted diet.

Lathyritic diet (*Fig.* 3).—Evidence of reduced growth rate was also observed in these animals. In addition, clefts developed in the proliferative and hypertrophic zones. The remnants of cartilage cell columns in this altered area contained abundant enzyme and the distribution and intensity of enzyme activity elsewhere was similar to that in the controls. The defective cartilage at the rib bone border is stabilized in some degree by the development of a shell of new

bone in the peripheral parts of the costochondral junction. In this area of new bone formation enzyme activity was particularly intense.

Liver

The staining reactions in experimental and control groups were essentially the same.

Chemical

The results are summarized in the table. There is a considerable random variation of activity between litters and also within each litter. In litters 1 and 3 no difference by any method of expressing liver activity could be found. In litter 2, significant differences were noted only when the results were expressed on a wet weight and 100 g. body weight basis. The nitrogen content of the livers of the lathyritic animals was significantly higher than that of the normal animals and thus there was no difference in the liver activity of the 2 groups of animals when expressed on a nitrogen basis. There is thus no clear evidence that lathyrism is associated with a regular alteration in liver succinic dehydrogenase activity.

TABLE.—*Succinic Dehydrogenase Activity of Livers of Normal and Lathyritic Rats*

Litter No.	Numbers of animals		Activity/g. wet weight (mean)		mg. nitrogen/g. wet weight (mean)		Activity/mg. nitrogen (mean)		Activity/100 g. body weight (mean)	
	Normal	Lathyritic	Normal	Lathyritic minus normal	Normal	Lathyritic minus normal	Normal	Lathyritic minus normal	Normal	Lathyritic minus normal
1	3	4	26·4	+0·3	26·9	+3·7	0·99	−0·11	127	+26
2	4	3	22·6	+8·3*	27·2	+6·5†	0·84	+0·10	120	+43†
3	2	2	35·6	−2·2	31·0	+1·6	1·15	−0·13	188	−18

* = $P < 0·01$.
† = $P < 0·05$.

DISCUSSION

In spite of a reduction in growth rate and the development of gross lesions in the cartilage matrix at the costochondral junction, no discernible histochemical changes in succinic dehydrogenase activity occur in the lathyritic animal. The

EXPLANATION OF PLATE

FIG. 1.—Normal, unrestricted diet. Costochondral junction. From left to right: cartilage bone border, hypertrophic, proliferative and resting zones. Enzyme activity (indicated by black dots in the cells) is present at all levels though less intense in the hypertrophic zone. (Unfixed frozen section about 10 μ stained for succinic dehydrogenase. No nuclear or cytoplasmic counterstain. × 170.)

FIG. 2.—Normal, restricted diet, Costochondral junction showing decreased depth of hypertrophic and proliferative zones. Distribution of enzyme similar to Fig. 1. Orientation, staining and magnification as for Fig. 1.

FIG. 3.—Lathyrism. (a) The hypertrophic zone at the extreme left is about half the normal depth. To the right is the zone of altered cartilage in which clefts separate remnants of the proliferative cartilage cell columns. Enzyme activity is present in all cells and in the clefts.
(b) Proliferative zone showing intense enzyme activity in the bony shell which develops in this region in lathyritic animals.
(c) Resting cartilage zone.
Orientation, staining and magnification as for Fig. 1.

1
2
3a
3b
3c

results of the chemical estimation of succinic dehydrogenase activity of the livers of normal and lathyritic animals also reveal no significant differences. There is thus no support from our results for the suggestion of Ramamurti and Taylor (1959) of a block in aerobic glycolysis in lathyrism, at least in so far as succinic dehydrogenase is concerned. This does not however preclude the possibility that a block may occur at some other point in the aerobic glycolytic cycle.

As regards the distribution of succinic dehydrogenase in healthy, normal, weanling rats, the present histochemical results indicate no preferential localization of enzyme in the hypertrophic cell region of actively ossifying costal cartilage as reported by Castellani and Zambotti (1954) and Whitehead and Weidmann (1959), the enzyme being present in the resting, proliferative and hypertrophic cartilage, though possibly less intense in the hypertrophic zone. This agrees essentially with the evidence presented by Hiatt and Shorr (1953) and Follis and Melanotte (1956). It should be noted that the method employed in the present study, using Nitro-BT as hydrogen acceptor, overcomes certain technical difficulties inherent in the 2,3,5-triphenyl tetrazolium chloride method of previous workers (Nachlas *et al.*, 1957).

SUMMARY

Weanling rats were fed on a diet containing semicarbazide hydrochloride in order to produce the well-defined skeletal lesions associated with lathyrism. The succinic dehydrogenase activity of the liver and rib cartilage of these animals was studied histochemically and the activity of the liver was also measured using a spectrophotometric method. No significant differences from normal were observed.

The enzyme was shown to be present throughout the resting, proliferative and hypertrophic cartilage.

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FACTORS AFFECTING THE CYTOTOXIC REACTION BETWEEN NEWCASTLE DISEASE VIRUS AND CELLS *IN VITRO**

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IT has been shown that large concentrations of Newcastle disease virus (NDV) produce toxic changes and destruction of several cell strains *in vitro* (Mason and Kaufman, 1960*a*). The reaction between the virus and these cells which caused cytopathic effects was obscure, since no evidence for virus multiplication was found during the production of cell changes (Mason and Kaufman, 1960*b*). It was important, therefore, to investigate the manner in which cellular alterations were induced by NDV.

The data in this report indicate that the cytotoxic action of NDV followed a rapid adsorption to cell receptors which are sensitive to the receptor destroying enzyme of *Vibrio cholerae* and that the toxic reaction which ensued was irreversible when immune serum was added 1–3 min. after virus. These and other experiments demonstrated certain similarities between this reaction and that described for haemolysis *in vitro* (Burnet and Lind, 1950; Sagik and Levine, 1957) and for the toxic action of NDV on cells *in vivo* (Moore, Diamond, Mackay and Sabachewsky, 1952; Prince and Ginsberg, 1957*a*; Ginsberg, 1951).

MATERIALS AND METHODS

Newcastle disease virus (NDV)

The California strain, 11914, of NDV was the principal source of virus for all experiments. The Hickman strain (obtained from Dr. H. S. Ginsberg) was also utilized in several experiments. Stock virus was prepared by allantoic inoculation of 9–10 day old embryonated eggs with 0·1 ml. of a 10^{-3} or 10^{-4} dilution of infected allantoic fluid. After 2 days' incubation at 35°, allantoic fluid was harvested, checked for sterility and stored in glass ampoules in a CO_2 box. Batches of stock virus were also checked for egg-infectivity, haemagglutinin titre and cytotoxicity for L cells.

Virus infectivity and haemagglutination

Infectivity titrations were carried out with serial tenfold dilutions of infected allantoic fluid diluted in Hanks' balanced salt solution (BSS), adjusted to pH 7·6. A volume of 0·1 ml. of each dilution was inoculated into the allantoic cavity of 8–10 day old embryonated eggs. After 3 days incubation at 35°, 0·5 ml. samples of allantoic fluid were removed from each

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egg and tested for viral haemagglutinins by the addition of an equal volume of 0·5 per cent chicken erythrocytes. In some cases allantoic fluids were diluted 1/16 and tested concurrently with the undiluted samples to ascertain that negative results were not the result of inhibitor present in the allantoic fluids (Chu, 1948). Tubes were observed after 1 hr. incubation at 4° and the 50 per cent infectious titre calculated using the method of Reed and Muench (1938). The infectious titre of any particular fluid is expressed as the number of 50 per cent egg infectious doses (EID_{50}) per ml.

To determine the virus haemagglutinin (HA) titre, 2-fold dilutions of material were prepared in PO_4 buffered saline (pH 7·4) in such a manner as to leave 0·5 ml. of the diluted virus in each tube. To each tube was added 0·5 ml. of 0·5 per cent thrice-washed chicken red blood cells. After incubation at 4° for 1 hr. they were read according to the pattern of the sedimented cells. The final dilution which produced " complete " haemagglutination was recorded as the titre and regarded as the dilution with one " HA unit " (HAU) of virus.

Cell strains

L strain cells were principally used in these studies. Four other cell strains consisted of U_{12} (human uterus, Swim and Parker (1955)), $LLCM_1$, (connective tissue of hypertrophied lymph node of mouse, Hull (1953)), Sarcoma 180, and KB (human epidermoid carcinoma, Eagle (1955)). L, U_{12} and $LLCM_1$ cells were maintained as stock cultures in this laboratory as described elsewhere (Mason and Kaufman, 1960*a*). S180 and KB strains were obtained as test tube cultures from Microbiological Associates, Inc., Washington, D.C.

Determination of grades of cytopathic effect

The method for determination and grading of the toxic alterations in cells has been described in greater detail elsewhere (Mason and Kaufman, 1960*a*). It consisted of the use of test tube cultures prepared from stock cultures of L, $LLCM_1$ or U_{12} cells or the tube cultures of KB and S180 obtained commercially. The number of cells per tube at the time of virus inoculation varied, but efforts were made to maintain this between $3–4 \times 10^5$ cells per tube. The supernatant medium which contained virus inocula consisted of 10 or 20 per cent heat inactivated (56°, 30 min.) horse serum and Solution 199, and is designated MS10 or MS20 respectively, in the text. Cultures were incubated at 35°. Virus-induced cell changes were observed microscopically and graded according to their severity from $\pm$ to 4+. The results in this report represent the average grade of cytotoxicity observed in 4 tubes inoculated with aliquots of the same dilution of material.

Immune serum

Immune serum was prepared by injecting rabbits intravenously with 10 ml. quantities of undiluted, infected allantoic fluid followed by 5–10 weekly intraperitoneal injections of 10 ml. each. The serum for control purposes was prepared in a similar manner with uninfected normal allantoic fluid. Sera from several rabbits were pooled and stored in a CO_2 box until assayed by the haemagglutination-inhibition technique using 0·5 ml. quantities each of 8 HA units of NDV, 0·75 per cent chicken erythrocytes and dilutions of immune serum. After 1 hr. incubation at 4°, the haemagglutination-inhibition titre (HAI), or the highest final dilution of immune serum which inhibited haemagglutination of cells by the virus varied from 1/1024–1/4096.

Receptor destroying enzyme (RDE)

Receptor destroying enzyme (obtained from Dr. J. Spizizen) was titrated by incubating 0·5 ml. quantities of serial 2-fold dilutions in Solution 199 (which contains 0·02 per cent $CaCl_2$) with 0·5 ml. of 0·75 per cent chicken red blood cells for 1 hr. at 37°. Further enzyme action was prevented by 2·5 per cent sodium citrate. Eight HA units of virus in 0·5 ml. amounts were added to each tube and, after thorough agitation, incubated at 4° for 1 hr. The amount of enzyme present in the last dilution of RDE which prevented subsequent virus haemagglutination was equal to " one unit ". The preparation used in this study had 1640 units per mg.

Other technical details will be described in connection with the individual experiments.

EXPERIMENTAL

Adsorption of virus at 0° and 35°

The adsorption of NDV by each of the 3 cell strains (L, $LLCM_1$ and U_{12} cells) was investigated. In addition to native stock virus, periodate treated virus was used in some of the experiments, since it has been reported that periodate treated NDV is adsorbed more effectively and elutes less readily from red blood cells (Bang and Libert, 1952; Hirst, 1949). The periodate treatment of virus consisted of mixing equal volumes of infected allantoic fluid and 0·014 M-sodium periodate (IO_4) at room temperature for 1 hr. followed by the addition of 20 per cent glucose ($\frac{1}{2}$ total volume). "Untreated" control virus was subjected to an identical procedure except for the substitution of saline in place of periodate.

One ml. of a 3·5 per cent suspension of cells washed twice with 10 ml. MS10 was mixed with 0·5 ml. aliquots of the virus samples at 35° or in an ice bath (0°) and incubated for 1 hr. Cells and supernatant were separated by centrifugation at 4° or room temperature in the case of virus-cell suspensions previously incubated at 35°. Table I contains the concentrations of viral haemagglutinin remaining in supernatants of suspensions of L, $LLCM_1$ or U_{12} cells after incubation for 1 hr. at 0° or 35° with periodate treated or untreated virus. The diminished haemagglutinin in the fluid of cell suspensions, as compared with control solutions without cells, shows that nearly complete or complete adsorption of virus was effected in suspensions of each of the three cell types. A comparable degree of virus adsorption occurred at either 0° or 35°. It was unnecessary to treat virus with sodium periodate to demonstrate adsorption to cells, but it appeared that the use of periodate treated virus assured the best definition of adsorption under such conditions.

To determine if virus eluted from cell suspensions at 35°, the packed cells remaining after adsorption experiments were resuspended in the same amount of Solution 199 and incubated at 35° for 2 hr. with occasional agitation. As shown in Table I (HA, eluate) supernatants from these cell suspensions did not

TABLE I.—*Adsorption of NDV Haemagglutinin by 3 Cell Types at 0° and 35°*

Virus	Cell type	Incubation temperature	HA titre before incubation	HA titre after incubation	Per cent virus adsorbed	HA titre (eluate)
NDV	No cells (control)	0	96*	96	0	—
		35	96	96	0	—
	L	0	—	6	94	6
		35	—	12	87	6
	U_{12}	0	—	6	94	6
		35	—	24	75	6
	$LLCM_1$	0	—	6	94	6
		35	—	12	87	<6
IO_4 NDV	No cells (control)	0	96	96	0	—
		35	96	96	0	—
	L	0	—	6	94	6
		35	—	6	94	<6
	U_{12}	0	—	6	94	<6
		35	—	6	94	6
	$LLCM_1$	0	—	<6	>94	6
		35	—	<6	>94	<6

* Cell suspensions in Solution 199, 35°, 2 hr. Values of supernatant.

contain significant amounts of haemagglutinating virus, regardless of the temperature used originally for virus adsorption, *i.e.*, 0° or 35°.

The data suggested a rather firm association between the adsorbed virus and cells, perhaps similar in some respects to the " stable union " developed between NDV and erythrocytes under conditions leading to haemolysis (Burnet and Lind, 1950 ; Burnet, 1950) and between NDV and chick embryo tissue culture cells (Levine and Sagik, 1956). Prince and Ginsberg (1957*b*) have also noted that NDV did not elute from Ehrlich ascites cell suspensions at 37°.

Time required for irreversible NDV-cell interaction

It was pertinent to investigate whether the adsorptive reaction was similar to that observed with red blood cells in which virus remains on the surface of erythrocytes (Anderson, 1947) or whether there was some degree of cell penetration. There is evidence that cell-adsorbed virus which cannot be acted upon by immune serum has penetrated into or through the cell membrane (Rubin and Franklin, 1957 ; Ackermann, 1958) ; consequently, experiments were conducted to determine whether immune serum could reverse the cytotoxic effect of virus. Experiments were also designed to determine after how long a period of virus action reversal by immune serum could be effected.

Test tube cultures of L cells received equal quantities of NDV-infected allantoic fluid and NDV-immune serum (total volume 1·5 ml.) added in the sequence shown in Table II. The results show that immune serum completely nullified the toxic properties of NDV if added 1 min. after infection, however, it was unable to prevent completely the cytotoxic effect of virus if 3 or more min. had elapsed. An identical experiment in which $LLCM_1$ cells were used indicated slight virus toxicity subsequently developed if one min. elapsed before the addition of immune serum. As the interval between virus addition and addition of immune serum increased to approximately 8 min., antibody was unable to modify the virus action and cytotoxicity increased to a maximum (4+).

TABLE II.—*Time Required for Irreversible Action of NDV on L Cells as Determined by Immune Serum Addition*

Time of addition of IRS in relation to NDV inoculation		Cytopathic effect* Days of incubation 1	3	5
5 min. before	NDV	0	0	0
Simultaneous with	NDV	0	0	0
1 min after	NDV	0	0	0
3 min. after	NDV	1+	1+	±
5 min. after	NDV	2+	2+	1+
8 min. after	NDV	4+	3+	4+
11 min. after	NDV	4+	4+	4+
Controls				
IRS + NAF (inoculated simultaneously) . .		0	0	0
NDV + NRS (inoculated 15 min. before NDV) .		4+	4+	4+

IRS = Immune rabbit serum.
NRS = Normal rabbit serum.
NAF = Normal allantoic fluid.

* Each figure is the average grade of cytopathic change obtained in 4 culture tubes.

These results imply that NDV was rapidly adsorbed by the cells and that reactions between the virus and cell surface ensued which rendered the virus insusceptible to antibodies. The time required for adsorption of toxic quantities of virus, as measured by the irreversibility of toxic changes with immune serum, is in good agreement with values obtained from studies of adsorption of this virus to other cells (Sagik and Levine, 1957; Moore *et al.*, 1952). The data also point to a difference from the lytic action of NDV on erythrocytes, since virus remains on the surface of the red blood cell preceding haemolysis and can be inactivated by antibody (Burnet, 1950). It would appear that the cytotoxic action of the virus involves cell penetration, at least to a position safe from antibody action. Evidence that similar events took place following adsorption of toxic NDV to Ehrlich ascites cells was obtained by using immune serum in a different manner (Prince and Ginsberg, 1957*b*).

Protection of cells from toxic virus action by RDE

The action of RDE on the sites for attachment of NDV to the surface of erythrocytes, preventing adsorption and haemolysis (Burnet and Lind, 1950) or on the surface of susceptible tissue cells, preventing infection (Stone, 1948) has been described. This suggested that similar attachment sites might be involved in the production of cytotoxic changes. This was investigated by treating cultures with RDE before introducing a challenging dose of toxic virus. Table III contains data from an experiment of this nature in which sets of tube cultures of L cells were exposed to various quantities of RDE or inactivated RDE (56°, 30 min.) for 4 hr. at 35° before inoculation with NDV-infected or normal allantoic fluid. It may be seen that pretreatment of the cells with 640 units of active, but not heat inactivated, RDE was able to prevent the appearance of virus-induced cellular changes. Sixty-four units of enzyme provided incomplete, but noticeable, protection for the cells. If less than 64 units of RDE were used toxicity developed to the same degree as in controls treated with heated RDE. Similarly designed experiments using U_{12}, KB and S180 cells confirmed that treatment of cultures with RDE in suitable quantities, ranging from 500–640 units per culture, prevented

TABLE III.—*Protection of L Cells from Cytopathogenic Action of NDV Infected Allantoic Fluid by Receptor-Destroying Enzyme (RDE)*

Treatment of cells	Inoculum	Cytopathic effect* Days of incubation 1	3	5
640 units RDE	NDV	0	0	0
	NAF	0	0	0
64 units RDE	NDV	2+	3+	3+
	NAF	0	0	0
6·4 units RDE	NDV	4+	4+	4+
	NAF	0	0	0
0·64 units RDE	NDV	3+	3+	4+
	NAF	0	0	0
640 units Heated 56° for 30 min.	NDV	4+	4+	4+
	NAF	0	0	0
Solution "199" RDE	NDV	4+	4+	4+
	NAF	0	0	0

* The final grade recorded for cytotoxicity of any virus dilution represents an average obtained from the values in four individual tubes.

virus damage. Data from all of these experiments indicated that intact RDE-sensitive receptors are necessary for NDV to induce toxic cell changes, just as these receptors are required for many other interactions between this virus and cells.

Indirect evidence that the toxic changes depended on a reaction between virus and the cell surfaces was obtained from experiments in which partial resistance to toxic, challenging doses was exhibited by cultures which had received high concentrations (1/2 dilution) of heat inactivated (56°, 30 min.) infected allantoic fluid. Cultures were also found resistant to a challenging dose of toxic virus if they were previously exposed to sub-toxic concentrations of virus. The data from many experiments involving all 5 cell types clearly indicated that sub-toxic inocula had reacted with the cells to render them resistant. It is not clear from these experiments whether the resistance caused by the first inoculum was due to receptor destruction, such as is produced by RDE, or whether virus contained in the sub-toxic inoculum set up blocking or interference to the challenging virus by virtue of action on the cell membranes.

The influence of sodium periodate on virus toxicity

Additional evidence that the virus and cell interaction preceding toxic damage is similar to that involved in the usual infectious process and haemolysis was obtained by modifying both tissue cell receptors and virus enzyme by sodium periodate. Sodium periodate modifies red cell receptor sites for influenza virus in such a manner that elution of adsorbed virus is greatly inhibited (Hirst, 1949). Likewise, periodate treatment of virus-infected allantoic fluid modifies the viral enzyme involved in adsorption so that the same result, inhibition of elution, is obtained (Bang and Libert, 1952). It has been shown that periodate treated influenza virus is still infectious for cells and that periodate treatment of tissue cells does not prevent infection by untreated virus (Fazekas de St. Groth and Graham, 1949). Using NDV, Bang and Libert (1952) reported that periodate did not interfere with haemolysis.

For these experiments, equal volumes of NDV-infected allantoic fluid or heated (56°, 30 min.) infected allantoic fluid and 0·014 M-$NaIO_4$ were incubated at room temperature for 30 min. Excess IO_4 was neutralized by 15–30 min. further incubation with a solution of 20 per cent glucose (1/2 total volume). Virus which had been so treated with periodate had altered enzyme, since there was no elution from erythrocytes and haemagglutination end-points remained stable after repeated cycles of resuspension and settling of cells at 37°. Under the same circumstances untreated virus was able to produce only weak or no haemagglutination. Tissue cell receptors were modified by adding solutions containing 0·25 to 1×10^{-3} M-$NaIO_4$ to cultures for 24 hr. Further action by this oxidizing agent was prevented by adding glucose (20 per cent) at the time of virus inoculation. These concentrations of periodate are similar to those used by Hirst (1949) to modify the receptors of erythrocytes.

The values of Table IV show that periodate treated NDV was as cytotoxic for KB and S180 cells as untreated virus. Maximal cytotoxicity was demonstrated by 1/2 dilutions of either virus preparation and the degree of alteration showed a direct relation to the dilution of virus. Similar results were also obtained for the action of periodate treated and untreated virus on L, $LLCM_1$ and U_{12}

cells. KB and S180 cells were also used in experiments in which the cells were treated with periodate and challenged with untreated virus. These studies demonstrated that the alteration of cell membranes produced by these concentrations of periodate did not interfere with the development of virus toxicity, at least for these 2 cell types, since the cytopathic effect was as great as that produced by virus in cultures of untreated cells. Collectively, the results indicate that periodate was unable to modify the cytolytic action of massive inocula of NDV and they provide additional evidence for a similarity between the virus-cell interactions resulting in tissue cell lysis, infection (Fazekas de St. Groth and Graham, 1949) and haemolysis (Bang and Libert, 1952).

TABLE IV.—*Comparison of the Toxicity of Untreated and Periodate Treated NDV for S*180 *and KB Cells*

Cell type	Virus treatment†	Cytopathic effect: Dilution of virus 1/6 — Days of incubation 1	3	5	1/30 — Days 1	3	5	1/60 — Days 1	3	5	1/600 — Days 1	3	5	1/6000 — Days 1	3	5
S180	NDV	3	3	3	2	2	2	1	1	1	±	0	±	0	0	0
	NDV-IO_4	3	3	3	2	2	2	1	1	1	0	0	0	0	0	0
	Heated NDV-IO_4*	±‡	±	±	0	0	0	0	0	0	0	0	0	0	0	0
KB	NDV	3	4	4	2	2	2	1	2	2	0	1	1	0	0	0
	NDV-IO_4	2	3	3	2	2	2	1	1	1	0	0	0	0	0	0
	Heated NDV-IO_4*	±	±	±	0	±	±	0	0	0	0	0	0	0	0	0

* NDV-infected allantoic fluid heated at 56° for 30 min. before IO_4 treatment.
† The virus used for this experiment had $10^{8 \cdot 7}$ EID_{50}/ml. and 800 HAU/ml.
‡ Non-specific, slight alterations, probably due to glucose concentrations.

Effect of temperature on cytotoxicity

Other experiments have demonstrated the influence of temperature on the interaction between NDV and red blood cells (Bang and Libert, 1952; Burnet, 1950). By comparison, little is known of the influence of temperature on virus toxicity. However, *in vivo* studies of tumour growth inhibition by massive inocula of NDV have shown that a reversal of the inhibitory effect can occur at temperatures which favour elution of virus from the cell (Prince and Ginsberg, 1957*b*). A comparison was made of the development of cytotoxic changes in L cells at 4° and 35°. No cellular alterations developed in cultures left at 4° for 5 days; however, if after 2 hr. at this temperature cultures were brought to 35°, destructive cell changes developed to the same extent as in those cultures incubated at 35° from the beginning of the experiment (Table V). The effect on the appearance of cell changes of replacing a majority of the toxic seed virus after 2 hr. incubation at 4° or 35° by fresh, virus-free medium was also investigated and the degree of cellular alterations induced in such culture sets are included in Table V. The results indicate that removal of the toxic seed after 2 hr. incubation at either 4° or 35° did not interfere with subsequent development of cytopathic changes at 35°. A comparison of the data obtained from these sets of treated and untreated cultures clearly shows that sufficient virus was adsorbed at 4° to produce toxicity even though visible cell damage did not ensue in cultures left at this temperature.

In other experiments which are not illustrated a comparison was made between the cytotoxicity which developed at room temperature (average 27° during experiment) and 35° using all 5 cell types. At room temperature cell damage was greatly delayed and was not observed until 3–5 days following inoculation. In many instances the degree of cellular alteration never reached the equivalent of that obtained at 35° after 1 day.

TABLE V.—*Effect of Temperature, 4° and 35° on Development of Toxicity of NDV‡ for L Cells*

Initial temperature (2 hr)	Treatment	Final temperature	Cytopathic effect† Days of incubation		
			1	3	5
35	None	35°	4	4	4
35	Washed*	35°	4	4	4
4	None	35°	4	4	4
4	Washed*	35°	3	4	4
4	None	4°	0	0	0
4	Washed*	4°	0	0	0

* Inoculum aspirated and 10·0 ml. of maintenance solution used to wash each culture.
† Each figure represents an average of the toxic changes occurring in 3 cultures.
‡ 1 : 2 dilution of NDV-infected allantoic fluid used to inoculate each tube.

Results from these experiments indicate that lower temperatures inhibit the development of cytotoxicity. This is similar to the influence of lower temperatures on the haemolytic activity of NDV; haemyolysis by NDV is greatly inhibited or abolished at temperatures near 4° and is considerably diminished by temperatures below 30° (Burnet and Lind, 1950; Sagik and Levine, 1957; Bang and Libert, 1952; Kahnke, 1951).

Relation of toxicity to haemagglutinin activity and infectivity

The similarities between the virus-cell relationship preceding cytotoxicity and during haemagglutination and the first stages of cell infection made it of interest to determine if one could obtain virus preparations which could execute one of these actions independently of the others. The rate of inactivation of these 3 virus manifestations by heat and ultraviolet light were investigated using L and U_{12} cells to indicate their effect.

The results obtained after heating NDV-infected allantoic fluid for various intervals at 56° and testing preparations for egg infectivity, haemagglutination and cytotoxicity are shown in Table VI. As the number of egg-infective doses in the samples decreased, cytotoxicity for L cells also decreased. Haemagglutinating capacity, on the other hand, was more stable toward heating and did not decrease in proportion to the decrease in cytopathogenic potency of the samples. These relations are summarized graphically in the Fig. in which the $\log_{10}$ reduction of egg-infective virus and virus haemagglutinin are plotted on the same graph with the $\log_{10}$ reduction of the highest dilution of virus which still produced 1+ cytopathic change (CPE titre). The response of U_{12} cells to samples of heated virus gave data similar to that obtained with L cells and suggested that the virus factors required for infectivity and cytotoxicity were closely associated whereas the haemagglutinating capacity was independent of either of these two properties.

TABLE VI.—*The Effect of Heating for Various Periods on Egg Infectivity Haemagglutination and Cytotoxicity for L Cells*

Inoculum	Time of heating at 56° (min.)	Log_{10} EID/ml.	Log_{10} HAU/ml.	Cytopathic effect* Dilution of seed 1/2 Days of incubation 1	3	5	1/5 1	3	5	1/10 1	3	5	1/100 1	3	5	1/1000 1	3	5
NDV	0	9·2	3·0	3+	4+	4+	2+	3+	3+	2+	2+	2+	1+	1+	1+	1+	1+	±
	3	8·3	3·0	2+	2+	2+	2+	2+	2+	1+	1+	±	±	±	±	0	0	0
	6	7·8	3·0	2+	1+	1+	1+	±	0	0	0	0	0	0	0	0	0	0
	10	7·5	3·0	1+	1+	±	±	0	0	0	0	0	0	0	0	0	0	0
	15	7·0	2·7	1+	1+	1+	1+	±	0	±	0	0	0	0	0	0	0	0
	20	5·0	2·4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	30	3·0	1·8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Each figure represents the average values of 4 cultures.

TABLE VII.—*The Effect of Ultraviolet Irradiation on Egg Infectivity, Haemagglutination and Cytotoxicity for L Cells*

Inoculum	Time of irradiation (min.)	Log_{10} EID/ml.	Log_{10} HAU/ml.	Cytopathic effect* Dilution of seed 1/2 Days of incubation 1	3	5	1/5 1	3	5	1/10 1	3	5	1/100 1	3	5	1/1000 1	3	5
NDV (Hickman)	0	10·0	2·7	3+	4+	4+	3+	2+	3+	1+	1+	1+	1+	1+	±	0	0	0
	1/2	8·6	2·7	3+	3+	2+	1+	1+	1+	1+	1+	1+	0	0	0	0	0	0
	1	6·2	2·7	1+	2+	2+	2+	1+	2+	1+	1+	1+	0	0	0	0	0	0
	2	4·3	3·0	3+	3+	3+	2+	1+	1+	1+	1+	1+	0	0	0	0	0	0
	10	2·0	3·0	2+	2+	3+	1+	1+	±	±	0	0	0	0	0	0	0	0
NAF	0	..	..	±	±	±	0	0	0	0	0	0	0	0	0	0	0	0
	3	..	..	±	±	0	0	0	0	0	0	0	0	0	0	0	0	0

* Each figure is the average of values from 4 cultures.

In contrast to the effect of heating, a dissociation of the cytotoxic factor from egg infectivity was obtained by treatment of infected allantoic fluid by ultraviolet light. For this experiment infected and normal allantoic fluids were exposed in open plates for various intervals to 6 germicidal units of a 30 watt G.E. ultra-violet lamp. Typical results of subsequent titrations of egg infectivity, haemagglutinin and cytotoxicity for L strain cells are shown in Table VII. With increasing periods of UV exposure there was a marked decrease of egg infectivity. Cytotoxicity, on the other hand, was altered much less by the longer periods of UV irradiation. It may be noted that virus samples with less than 10^5–10^6 EID/ml. were capable of producing toxicity, and these concentrations of infective particles in inocula of untreated virus were insufficient to produce comparable cell damage. The divergence of egg-infectivity and cytotoxicity made it evident that the viral moieties responsible for these 2 properties are not identical and that cytotoxicity may be produced by virus which is not fully infectious. As

with heat, partial inactivation of virus by ultraviolet light showed that haemagglutinating capacity was more resistant to this agent.

A dissociation of cytotoxicity from both haemagglutinating capacity and infectivity had also been demonstrated by virus strains which received cyclic passages through eggs and L cell cultures (Mason and Kaufman, 1956). *In vivo* experiments with NDV and Ehrlich ascites tumour (Prince and Ginsberg, 1957*a*) and *in vitro* experiments with influenza virus and HeLa cells (Henle, Girardi and Henle, 1955) have shown that loss of egg-infectivity and of cytotoxicity do not take place concurrently and it would seem apparent from these studies as well as those reported here that toxicity may indeed be manifest by non-infectious virus.

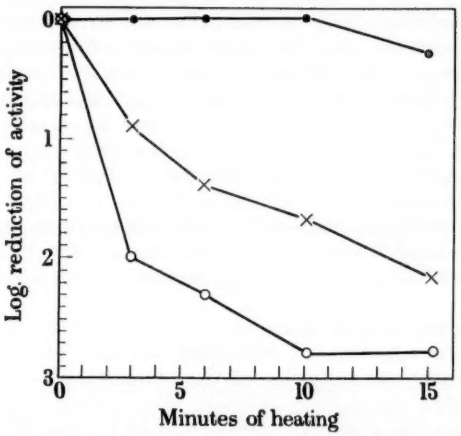

FIG.—Comparison of the rate of reduction of cytotoxicity, egg infectivity and haemagglutination by heat (56°).

○———○ Cytopathogenicity (CPE titre).
×———× Egg infectivity (EID_{50}).
●———● Haemagglutination.

DISCUSSION

Virus toxicity in the sense referred to in this report affects cells only upon the introduction of a relatively large concentration of virus. The ensuing cell damage, which occurs without the usual evidence of concomitant virus replication, raises the question of whether a different type of association occurs between the virus and cell. It was suggested by other investigators (Moore *et al.*, 1952) that tumour inhibition due to high concentrations of NDV was the result of a surface reaction with cells (Ehrlich ascites carcinoma) and that the entire process resembled the reaction occurring between this virus and erythrocytes which results in haemolysis. That actual inhibition of tumour growth in this system is accompanied by intracellular reactions was subsequently demonstrated by Prince and Ginsberg (1957*a*, 1957*c*). At the same time, results of certain experiments show that similarities exist between the initial steps of virus contact with red blood cells resulting in haemolysis and virus contact with Ehrlich ascites cells resulting in lysis. Experimental data in this report also suggest a similarity between the initial reactions

leading to NDV haemolysis and cytotoxicity. The evidence for this, with references to indicate corresponding data regarding NDV haemolysis, consists of the following: (1) Haemolysis and toxicity are favoured by a multiplicity of virus per cell (Burnet and Lind, 1950; Sagik and Levine, 1957; Bang and Libert, 1952). (2) Both haemolysis and cytotoxicity involve adsorption of virus to the surface of cells which can occur at 4° or 37°. Elution of virus at 37° does not occur readily (Sagik and Levine, 1957; Bang and Libert, 1952), though the latter authors do not wholly agree with Burnet's (1950) view that firm virus-cell union is necessary for haemolysis. (3) Adsorption of haemolytic and cytotoxic virus takes place rapidly, within a matter of minutes (Sagik and Levine, 1957). (4) Pretreatment of tissue cells or red blood cells with adequate concentrations of receptor destroying enzyme renders them insusceptible to virus toxicity and haemolysis (Burnet and Lind, 1950). Likewise, pretreatment of erythrocytes or tissue cells with non-haemolytic or non-cytotoxic virus interferes with subsequent virus action (Burnet and Lind, 1950). (5) Neither cytotoxicity nor haemolysis are inhibited by sodium periodate treatment of virus or of cells (Bang and Libert, 1952). (6) Neither haemolysis nor cytotoxicity proceed at temperatures near 0° and they are greatly inhibited by temperatures below 30° (Burnet and Lind, 1950; Sagik and Levine, 1957; Kahnke, 1951). (7) Heat destroys both the haemolytic and cytotoxic activity of the virus before haemagglutinating activity is lost (Burnet and Lind, 1950; Granoff and Henle, 1954), but others maintain (Sagik and Levine, 1957) that haemagglutinating and haemolytic powers are equally heat-labile.

So far as comparison can be made, the virus-cell reactions preceding the cytotoxic and haemolytic activities of NDV are similar. Moreover, evidence from other sources regarding the influence of the above factors on infectivity, in the absence of immediate toxic changes, suggests that the initial steps in the virus-cell reaction are influenced in a similar manner except for the dependence of toxicity on a large inoculum.

One major difference between NDV haemolysis and cytotoxicity is the evidence that virus antigen disappears from the cell surface. This was suggested from the study of 2 cell types (L and U_{12}) in which immune serum was unable to abolish the cytotoxic effect of virus which remained in association with cells for longer than 1–3 min. The inaccessibility of cytotoxic virus to immune serum provides a sharp contrast to the association of virus with erythrocytes in the haemolytic process in which immune serum prevents lysis by inactivating virus which remains on the cell surface (Burnet and Lind, 1950; Burnet, 1950). Rather, the reactions leading to cytotoxicity resemble those of susceptible host cell infection (Rubin, 1957) in which NDV also rapidly becomes inaccessible to the neutralizing action of immune serum. There is evidence to indicate that this represents actual virus penetration of the cell membrane (Ackermann, 1958) and it would seem correspondingly valid to reason that the toxic cell response also occurs after virus penetration of the cell.

So far as data may be compared, no major differences are suggested between the toxic action of NDV on cells *in vitro* and the toxic action of NDV on Ehrlich ascites cells *in vivo* (Moore *et al.*, 1952; Prince and Ginsberg, 1957*a*) or the pneumotoxicity of NDV in mice (Ginsberg, 1951). If one compares the toxic action of NDV with that of influenza virus, the process of cell damage is very similar in the 2 cases (Henle *et al.*, 1955). The major exception is that non-

infectious haemagglutinins are produced during cytopathic changes resulting from influenza virus and not during similar cell damage by NDV (Mason and Kaufman, 1960*b*; Henle *et al.*, 1955). This difference, however, is probably only in the degree of maturity of virus products elaborated by the cells under these circumstances, since there is recent evidence for development of relatively immature incomplete forms of NDV during cytopathic changes in Ehrlich ascites cells (Prince and Ginsberg, 1957*c*).

The protection against virus toxicity given to L, U_{12}, KB and S180 cells by RDE is similar to the protection it offers red blood cells from haemolysis by NDV (Burnet, 1950) and Ehrlich ascites carcinoma from lysis by NDV (Prince and Ginsberg, 1957*a*; Moore and Diamond, 1953). It has been reported recently (Wagner, 1955) that the treatment of HeLa cultures with RDE did not protect the cells from toxic inocula of influenza virus. However, these results are not necessarily contradictory to those of this report, since a smaller quantity of RDE (100 units) was used to treat each culture. For complete protection it was necessary to treat the 4×10^5 cells (approximate) in tube cultures of L, U_{12}, KB and S180 cells with 500–640 units of enzyme. The massive amount of virus used to test for removal of attachment sites could explain the need to use a large amount of enzyme in these experiments. A recent investigation (Kodza and Jungeblut, 1958) has stated that L cells do not adsorb RDE. The fact that encephalo-myocarditis (EMC) virus was used to titrate RDE adsorption may explain the discrepancy between the conclusions from this work and experiments reported here.

SUMMARY

Factors influencing the cytotoxic reaction between massive inocula of Newcastle disease virus and cell strains *in vitro* were investigated. Data indicated that the virus was adsorbed to cells at both 4° and 35° and that insignificant elution of virus occurred after 2 hr. at 35°. The toxic effect of virus was not reversible by immune serum after 1–3 min., suggesting that further reactions, perhaps intracellular, took place. Toxic changes and presumably virus adsorption were prevented by adequate amounts of RDE or pretreatment of cells by certain non-toxic samples of virus. Periodate treatment of either virus or cells had no effect on the subsequent development of toxicity, but the appearance of toxic changes was greatly inhibited or abolished by lower temperatures. Degrees of virus inactivation by heat and ultraviolet light disclosed that haemagglutination could occur with preparations that were non-toxic and that preparations with diminished infectivity could still produce cytotoxic changes, demonstrating the individuality of the viral components responsible for these properties.

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PAPER ELECTROPHORESIS OF SERUM LIPOPROTEINS IN THE NORMAL AND HYPERCHOLESTEROLAEMIC RAT

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Not much has been written about the electrophoretic pattern of serum lipoproteins in the rat. Morris and Courtice (1955) found it difficult to obtain good separation of the serum lipoproteins in this animal. Filios, Andrus, Mann and Stare (1956) and Portman, Lawry and Bruno (1956) mention that their experimental diets produced an increase in rat β-lipoproteins, but make no comment on the normal lipoprotein spectrum. Wilgram, Lewis and Best (1957) observed an increase in the low-density and α-lipoproteins of rats given choline supplemented, cholesterol-rich, diets, but the average maximum serum cholesterol level achieved in their study was only 128·1 mg. per cent.

This study is an account of the normal and abnormal lipoprotein patterns found in the rat throughout a wide range of serum cholesterol levels, with observations on the possible functional importance of such changes.

MATERIALS AND METHODS

Double-hooded Norwegian and male albino rats weighing 250–350 g. were used in the course of this work. The data reported here were obtained during a group of miscellaneous studies including work on the tissue mast cell (Watson, 1958 and 1961) and certain dietary experiments. Over 200 animals have been used and the experimental data have been consistently reproduced at different times.

The basic lipogenic diet throughout was normal rat bran plus 2 per cent cholesterol, 2 per cent. sodium cholate and 0·3 per cent methyl thiouracil. Lower cholesterol levels were obtained by reducing the concentrations of cholesterol and cholate, higher levels by the addition of corn oil or cottonseed oil. The method of cholesterol estimation used initially was that of King (1951), and latterly that of Zlatkis, Zak and Boyle (1953) as modified by Henly (1957). Lipoprotein electrophoresis was carried out as previously described (Watson, 1957) the method being based on that of Swahn (1952) using Sudan Black B as staining agent. For protein electrophoresis smaller amounts of serum (0·02 ml.) were used and the stain was bromophenol blue.

The distribution of cholesterol between different lipoprotein bands was estimated by a simple elution procedure. A marker strip was cut from the blank electrophoresis paper and the lipoproteins developed. Areas of blank paper corresponding to the lipoprotein bands were cut up with scissors and eluted overnight in the Zlatkis ferric chloride : acetic acid reagent. A portion of serum free paper was used as blank. 5 ml. aliquots of eluate were subsequently developed with concentrated sulphuric acid as in the method for total cholesterol.

RESULTS

A typical example of rat serum protein electrophoresis with the corresponding human pattern is shown in Fig. 1. There are 5 main bands—γ, β, α-1 and -2 globulins and albumin. A faintly staining pre-albumin component is sometimes seen. The relative amounts of each component vary somewhat from human serum. There is less gamma, and slightly more beta, globulin. α-2 globulin is very scanty.

Albumin and α-1 globulin run slightly behind their human counterparts. β globulin lies in approximately the same position, but γ globulin runs further than human gamma so that the γ and β globulins of rat serum are not always clearly separate, but often appear as differently shaded components of one broad band. These findings compare closely with the mobility data for rat serum given by Deutsch and Goodloe (1945) in their Tiselius survey of 20 animal species.

The range of cholesterol levels in the sera studied was from 50 mg. per cent to over 2000 mg. per cent. Fig. 2 shows the lipoprotein pattern of normal rat serum cholesterol level 72 mg. per cent. There is little lipid staining material present, but it is quite well differentiated and most of it occurs as α-lipoprotein. In addition two fainter bands are seen, one associated with β globulin (the β-lipoprotein band) and another running ahead of α-lipoprotein, a pre-albumin band corresponding to the pre-albumin protein zone. Fig. 3 shows what happens when as a result of dietary manipulations the serum cholesterol rises. A new lipo-protein band appears which increases in density with the increasing cholesterol concentration. This band is distinct from β-lipoprotein. It lies between the β-globulin and albumin bands and may be associated with α-2 globulin. It is remarkable how little protein there seems to be in relation to it. In this respect it is similar to human pre-β-lipoprotein. In some sera slight increases in the α-lipoprotein were noted, but were trivial compared with the emergent, provisionally named α-2 lipoprotein.

Elution analysis showed that in normal rats about 80 per cent of the serum cholesterol was located in α-lipoprotein. Fig. 4 shows the cholesterol distribution in one serum in which the total cholesterol was 630 mg. per cent. Only 5 per cent is in α-lipoprotein, 82 per cent being in the new α-2 lipoprotein, 10 per cent in β lipoprotein and 3 per cent in the chylomicron zone. With higher serum cholesterol levels even larger percentages are found in α-2 lipoprotein. It seems then that this new lipoprotein band contains most of the extra cholesterol in the serum. The amount in β-lipoprotein is greater than normal and there is probably a reduction in the absolute quantity of cholesterol carried by α-lipoprotein. It is obvious that α-2 lipoprotein is deficient in protein and overloaded with cholesterol and theoretically might be considered an "unstable lipoprotein". Spontaneous confirmation of this supposition occurred as follows.

The majority of normal sera if kept in sterile bottles at 4° will give reproducible lipoprotein patterns over a period of weeks and sometimes for months. However, after the hypercholesterolaemic rat sera has been in the refrigerator, in some cases for as little as 5 days, the clear samples became turbid and those which were already slightly turbid became milky. The rate and degree of change was generally related to the height of the cholesterol level. Repeat electrophoresis of such sera invariably revealed a fundamentally different lipoprotein pattern (Fig. 5). α-2 lipoprotein had disappeared and instead a dense lipid band had appeared just ahead of the application line. Elution analysis showed a redistribution of the cholesterol. For the serum shown in Fig. 4 the new figures were 85·5 per cent in the new lipid band, 11·5 per cent in β-lipoprotein and 3 per cent in α-lipoprotein.

DISCUSSION

It seems reasonable to infer from these experimental data that hypercholesterolaemia in the rat is associated with the formation of an abnormal and unstable

lipoprotein which on electrophoresis runs to a position between β-globulin and albumin and perhaps corresponding to the α-2 globulin zone. It shows *in vitro* instability. The increase in serum turbidity suggests a change from colloidal to an emulsion phase with physical separation of lipid from protein. Certainly it no longer occupies the same position on paper electrophoresis but although its new position is in the chylomicron zone the large cholesterol content of the lipid band indicates that it is not composed of typical chylomicron particles which have a very low cholesterol content (Bragdon, Havel and Boyle, 1956).

A lipoprotein which is so unstable *in vitro* may also be unusually unstable *in vivo*. This could partly or wholly explain the ease with which tissue and vascular lipidosis is produced in the rat when really high serum cholesterol levels are obtained. Wissler, Eilert, Schroeder and Cohen (1954) attributed intimal lipidosis to the high cholesterol : phospholipid ratio in their lipaemic rats. Lipoprotein stability depends on " the mosaic interlacing of ionic phospholipids and protein molecules on the surface of the lipoprotein " (Oncley, Gurd and Melin, 1950). If both protein and phospholipid are inadequate relative to the amount of non-ionic lipid (in this case cholesterol) requiring transport, the greatest possible degree of surface instability of the lipoprotein must presumably exist.

A number of authors have reported on the rat lipoprotein pattern in hypercholesterolaemia (Filios *et al.*, 1956; Portman *et al.*, 1956; Rohatgi and Banerjee, 1959; Sesskind, Wheatley, Rasmussen and Wissler, 1959) but all of these without exception described the main change as an increase in the β-lipoprotein fraction. There may be a slight increase in the β band but this is trivial compared with the emergence of a new and uncharacteristic lipoprotein. We have already reported a similar phenomenon in the chicken (Campbell, Church, Dick, Lawrie, Pirrie and Watson, 1959) and the pre-β-lipoprotein of human serum may be another manifestation of a similar kind, for this lipoprotein characteristically appears in the zone between β and α-2 globulin, and is singularly deficient in a protein component.

The presence of an abnormal electrophoretic lipoprotein as demonstrated here is implicit in the work of Lewis, Green and Page (1952). Using a high density system of ultracentrifugal analysis (the $-S$ system at S.G. 1·21) they showed that gross hypercholesterolaemia in the rat results in the appearance and progressive increase of low density lipoproteins in the ranges $-S > 70$ and $-S$ 40 to 70. These are outside the $-S$ range (25–35) which corresponds to the β-lipoprotein of paper electrophoresis.

There seems to be a limit to the lipid binding or lipid carrying capacity of the serum proteins, and this varies in different species. When it is exceeded the excess lipid (often cholesterol) is maintained in a colloidal phase with phospholipid as the main stabilizing agent and a small amount of protein, not usually utilized for the purpose, is incorporated to give the functional structure of a lipoprotein. The stability of such a lipoprotein is probably minimal however and easily disrupted in tissue by endogenous enzymes. In œstrogen treated hypercholesterolaemic chickens there is a real increase in α-2 globulin in association with the emergent α-2 lipoprotein (Campbell *et al.* 1959). This phenomenon was not observed in the non-œstrogen treated hypercholesterolaemic chickens and did not occur in the rats studied here. It would be interesting to observe whether œstrogen treatment of the rat in similar circumstances produces an increase in the rather scanty α-2 globulin of this animal.

SUMMARY

In normal rat serum α-lipoprotein predominates and β-lipoprotein is relatively scanty.

During experimental hypercholesterolaemia the main lipoprotein modification is not an increase in β-lipoprotein but the appearance of an unstable α-2 lipoprotein which is deficient in protein.

The functional significance of this fact is discussed in some detail.

I wish to thank Professor L. J. Davis for his encouragement and interest in this work, and Messrs. Boots Pure Drug Company, Limited, for dietary constituents.

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EXPLANATION OF PLATES

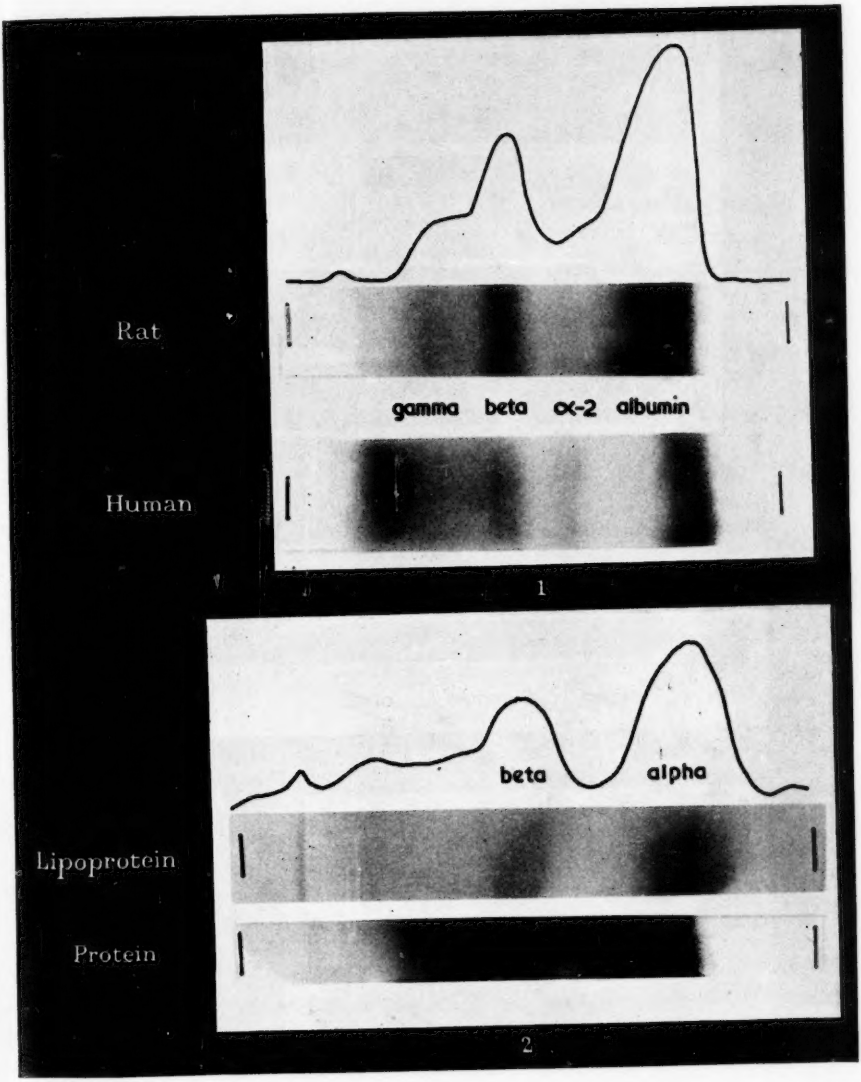
Fig. 1.—Protein electrophoresis of normal rat serum. The human pattern is shown alongside for comparison. α-1 globulin is incorporated in the albumin trailing edge.

Fig. 2.—Lipoprotein electrophoresis of normal rat serum. α-lipoprotein includes a pre-albumin component.

Fig. 3.—An α-2 lipoprotein emerges and increases in amount as the serum cholesterol rises. There is little stainable protein associated with it.

Fig. 4.—Marker strip showing the distribution of cholesterol among the lipoproteins of rat serum with a cholesterol level of 630 mg. per cent.

Fig. 5.—Lipoprotein pattern of fresh hypercholesterolaemic rat serum and after 5 days at 4°. α-2 lipoprotein has disappeared and a new lipid staining band has appeared just ahead of the application line.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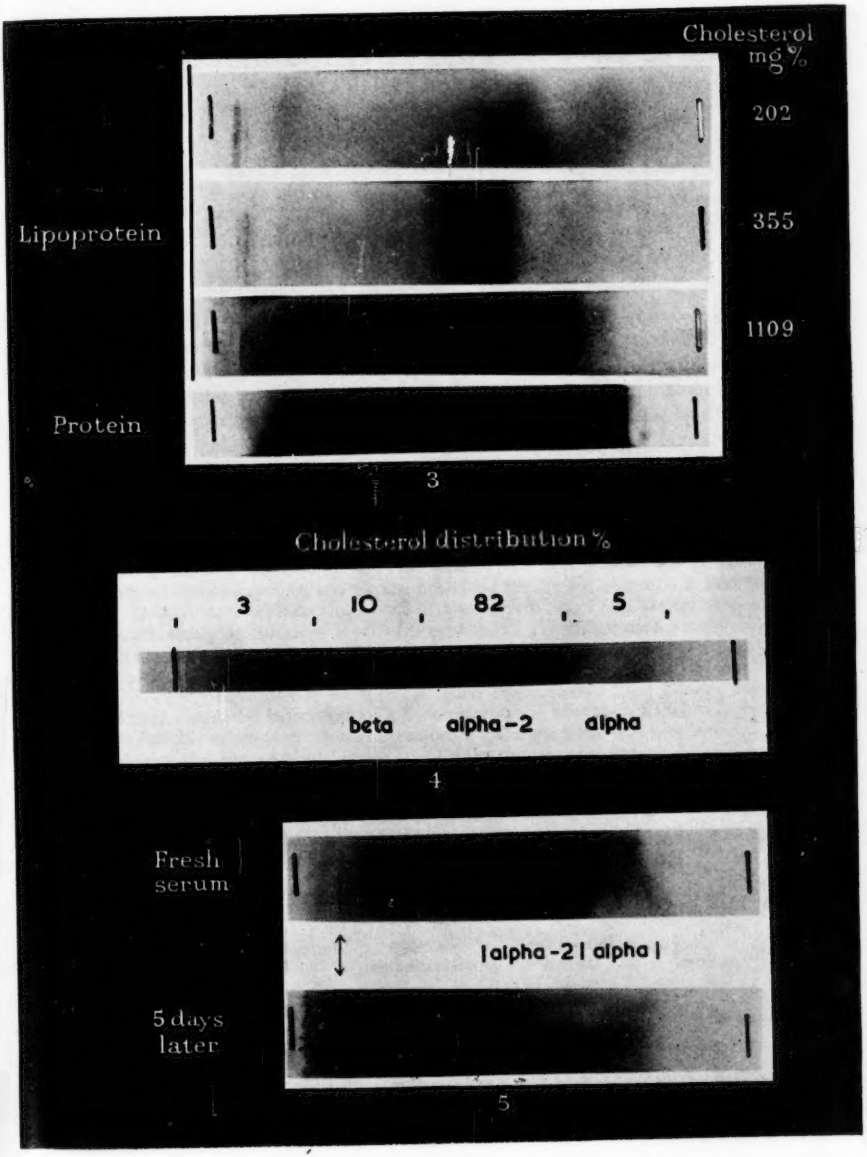
Cholesterol mg %
202
355
1109
Lipoprotein
Protein
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Cholesterol distribution %
3
10
82
5
beta
alpha-2
alpha
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Fresh serum
alpha-2 | alpha
5 days later
5

A CALCIFYING CARDIOPATHY PRODUCED BY STRESS AND CALCIUM ACETATE

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In a previous paper (Selye and Prioreschi, 1960), we described an electrolyte-steroid-induced cardiopathy characterized by calcification (ESCC) in the myocardium, coronary arteries, and kidney. This syndrome was produced by administering 9 α-fluorocortisol and calcium acetate to rats exposed to stress.

The purpose of this paper is to show that under suitable conditions, a similar cardiopathy can be produced, even without exogenous corticoids, by the combined action of stress and calcium acetate.

MATERIALS AND METHODS

Sixty female Sprague-Dawley rats from the Holtzman farm, with a mean initial body weight of 96 g. (range: 90–110 g.), were divided into 6 equal groups and treated as indicated in the Table. As a stressor we used forced restraint, which consisted in fastening the animal to a board with adhesive tape, in the prone position, for 24 hr. (Groups 2 and 3) or 5 hr. (Groups 5 and 6). Calcium acetate was given at the beginning of the experiment in 3 ml. of water, by stomach tube, at the dose level of 2 or 4 mM; Groups 1 and 3 received an additional gavage 8 hr. later, all other conditions being equal.

The experiment lasted 24 hr. for Groups 1–3, and 8 hr. for Groups 4–6. At the end of these periods all surviving animals were killed with chloroform. The hearts, kidneys and stomachs were first inspected by means of a binocular loupe and then fixed in alcohol-formol (Renaud, 1959) for the subsequent demonstration of calcium with the von Kóssa technique. The mean grades of the myocardial lesions, nephrocalcinosis, and gastric lesions were based on examination of the entire freshly dissected organs with a binocular loupe, while the grades of "coronary calcification" were based on microscopic readings. The severity of the lesions was assessed in terms of an arbitrary scale of 0–3 (0 = no lesions, 1 = barely detectable lesions, 2 = moderate lesions, 3 = most severe lesions); the mean grades are listed with standard errors in the Table.

TABLE.—*Production of Cardio-Renal Lesions by Calcium Acetate and Stress*

Group	Treatment	Myocardial lesions	Coronary calcification	Nephrocalcinosis	Gastric lesions	Mortality per cent
1	Calcium acetate 2mM × 2	0	0	0	0·	
2	Restraint 24 hr.	0	0	0	1·1 ± 0·20	0
3	Calcium acetate 2mM × 2 + restraint 24 hr.	2·4 ± 0·30	2·0 ± 0·45	1·7 ± 0·30	2·8 ± 0·17	60
4	Calcium acetate 4mM × 1	2·0 ± 0·43	1·0 ± 0·45	1·5 ± 0·25	3·0 ± 0	10
5	Restraint 5 hr.	0	0	0	0·4 ± 0·17	0
6	Calcium acetate 4mM × 1 + restraint 5 hr.	2·0 ± 0·45	1·7 ± 0·27	1·2 ± 0·38	3·0 ± 0	90

* Fellow of the Life Insurance Medical Research Fund.

RESULTS

The lesions were typical of the ESCC ; macroscopically, the heart often had a " tigroid " appearance, because of the distribution of calcification in parallel lines. The coronary arteries showed sclerosis of the Mönckeberg-type (Fig.). In the kidneys, necrosis and calcification affected the proximal convoluted tubules of the cortex preferentially. In the animals given calcium acetate, and especially in Groups 3, 4 and 6, the ventriculus (but not the proventriculus) showed a deeply penetrating, necrotizing gastritis, while in the rats subjected to restraint alone

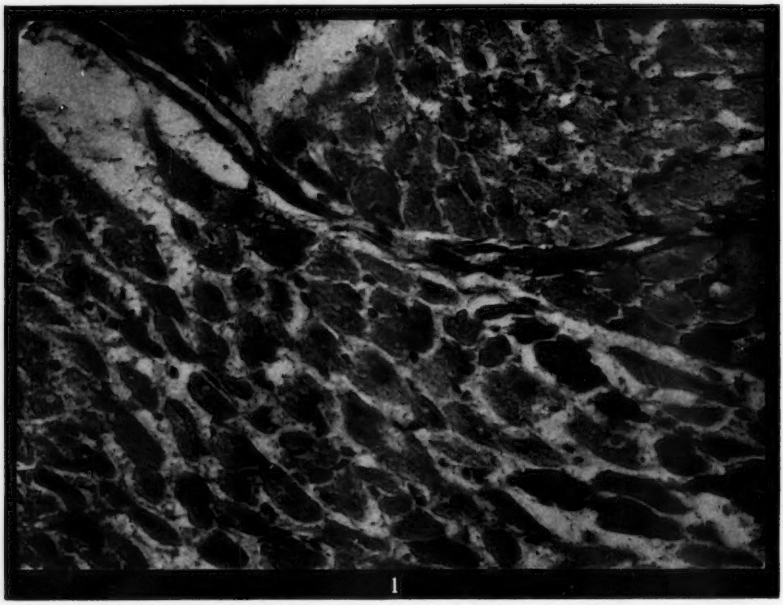

FIG.—Calcification of myocardial fibres and coronary sclerosis of the Mönckeberg-type (von Kóssa × 420).

(Groups 2 and 5), the gastric lesions consisted only of the usual, limited, haemorrhagic erosions characteristic of the alarm reaction.

As shown in the Table, cardio-renal lesions were obtained only in animals treated with either 4 mM of calcium acetate (with or without restraint) or 2 mM of this salt plus restraint. When given alone, both the stressor and the lower dose level of the salt were completely ineffective.

DISCUSSION

The role of stress in the production of this experimental cardiopathy seems to be fundamental. Restraint was necessary for the production of pathological changes in the calcium acetate-treated rats of Group 3 (2 mM twice), while the animals

given this dose of the electrolyte alone (Group 1) showed no noteworthy cardiac, renal or gastric lesions. The rats of Group 4 were given the same amount (4 mM) of calcium acetate as those of Group 1, but in 1 dose instead of 2 ; this method of administration resulted in a very intense necrotizing gastritis often accompanied by thrombosis of the splenic vessels and consequent infarction of the spleen. In these rats, stress was so intense that severe cardiac lesions ensued without any additional treatment. The intensity of these lesions could not be further aggravated by the additional stress of restraint (Group 6), which, in this case, increased the mortality rate from 10 per cent to 90 per cent (cf. Groups 4 and 6).

Although investigations on a necrotizing type of experimental cardiopathy (electrolyte-steroid-induced cardiopathy with necrosis—ESCN) (Selye, 1958) have proven that the cardiotoxic effect of stress is not mediated through the adrenals (Selye, Prioreschi and Jean, 1960), only further studies in adrenalectomized animals will show if this is also the case for the calcifying lesions described here.

Like the other experimental syndromes described elsewhere—the electrolyte-steroid-induced cardiopathy with hyalinization (ESCH) (Selye, 1958), the ESCN, and the ESCC—the cardiopathy described here seems to be pluricausal. Its development depends, not upon a single pathogen, but upon the creation of complex pathogenic situations in which stress and " adaptive hormones " play particularly important roles.

SUMMARY

Calcifying cardiovascular lesions can be produced by the administration of calcium acetate with or without restraint. The cardiopathy is characterized by calcified streaky necroses (" tigroid ") of the myocardium, and coronary sclerosis of the Mönckeberg-type ; it is usually accompanied by nephrocalcinosis. Stress appears to play a decisive role in the production of such lesions.

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THE DEMONSTRATION OF LYSOZYME AS A DOMINANT TUBERCULOSTATIC FACTOR IN EXTRACTS OF GRANULOMATOUS LUNGS*

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IT is well known that specific antibody plays at most only a secondary role in acquired resistance to tuberculosis. However, many chemical compounds which possess inhibitory activity for tubercle bacilli *in vitro* have been obtained from animal tissues and fluids. (Björnesjö, 1952*a*, 1952*b*; Dubos and Hirsch, 1954; Patnode, 1954; Myrvik and Weiser, 1954; Oshima, Fujita, Takeoka and Tsuji, 1958; Franc, 1958; Myrvik and Soto-Figueroa, 1958; Fujita, 1959; Nakazima, 1959.)

The possibility that lysozyme might play a role in resistance to tuberculosis emerged from the studies of Myrvik and Weiser (1951) who reported that lysozyme levels were increased in the sera of rabbits after vaccination with large doses of BCG and Bayol F. They demonstrated also that lysozyme had inhibitory effects on the growth of tubercle bacilli *in vitro*. Studies which revealed large amounts of lysozyme in the alveolar macrophage (Myrvik, Leake and Fariss, 1960) provided a possible explanation for the origin of the serum lysozyme in rabbits undergoing a granulomatous response induced by BCG. These observations prompted a reinvestigation into the nature of the granulomatous response in the lung. The aim was to assess the possible role of lysozyme in acquired resistance to tuberculosis.

This communication is concerned with an experimental model for the production of a diffuse granulomatous type of response in the lungs of rabbits. This technique provides a cellular environment simulating that occurring in reinfection tuberculosis. The data indicate that extracts of such granulomatous lungs possess considerably more tuberculostatic activity than extracts from normal rabbit lungs. Lysozyme is one of the main tuberculostatic factors in these extracts.

MATERIALS AND METHODS

Technique for producing granulomatous response in the lung

Rabbits of the New Zealand strain (weight 1·8–2·2 kg.) were vaccinated with 50 mg. of heat-killed BCG (dry weight) suspended in Freund's adjuvant (Freund, 1946) in 2 sites in the inguinal region, each containing 25 mg. Approximately 6 weeks after vaccination, 5 mg. of BCG suspended in saline were injected intravenously. The rabbits were killed and the lungs were removed four days after the intravenous challenge. The characterization of the granulomatous response in such lungs will be published elsewhere (Myrvik, Leake and

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Oshima, unpublished). Control groups of animals included normal, vaccinated-unchallenged and unvaccinated-challenged rabbits.

Preparation of lung extract

The lungs were trimmed free of the trachea, heart, and excess fat and connective tissue. The lungs were weighed and then homogenized with 2 vol. of water (1 : 3 dilution) in a Waring Blender for 2 min. The homogenate was adjusted to pH 3·0 with HCl and allowed to extract overnight at 4°, after which the homogenate was readjusted to pH 7·0 with NaOH. The extract was recovered by centrifuging the homogenate for 20 min. at 3,000 *g*. The clear amber coloured extract was sterilized by filtration and stored at −10°.

Bacteriostatic assay

The fractions to be tested were serially double diluted in 0·5 ml. quantities of sterile distilled water in screw cap test tubes. A modified Dubos medium (Dubos and Middlebrook, 1947) was used in which the following constituents were omitted: casein hydrolysate, $CuSO_4$, $ZnSO_4$, and $CaCl_2$. Sterile albumin solution—Fraction V, Pentex (Pentex, Inc., Kankakee, Illinois)—was added to the medium just before use to give a concentration of 0·5 per cent. The final medium was dispensed in 0·5 ml. quantities in the sets of tubes containing the diluted fractions. This resulted in halving the concentration of the medium to give a final concentration of albumin, 0·25 per cent, and Tween 80, 0·02 per cent.

Mycobacterium phlei and the BCG strain of *Mycobacterium bovis* were grown on Proskauer-Beck medium and were used as assay organisms. The bacterial suspensions were prepared by grinding a portion of the surface pellicle in a sterile mortar and suspending it in modified Dubos medium (without albumin). Large clumps were removed by low speed centrifugation. The turbidity was adjusted to give approximately 250 nephelos units in a Coleman model 9 nephelometer. Each tube was inoculated with one drop (0·02 ml.) delivered from a standard bore capillary pipette.

Assays inoculated with BCG were incubated for 4–6 days and assays inoculated with *Myco. phlei* were incubated for 2–4 days at 37° and examined for macroscopic growth. The endpoint was the highest dilution of the test material which gave complete inhibition of growth of the test organism.

Column chromatography

Columns of Cellex-CM cation exchange cellulose were used (Cellex CM, Cation exchange cellulose, exchange capacity of 0·6 milliequivalents per gram, Type: carboxymethyl exchange groups on Whatman cellulose (CM-W). Bio-Rad Laboratories, Richmond, Calif.). A column (2·0 × 9·0 cm.) made with 5 g. of cellulose powder would completely remove all tuberculostatic activity present in 50 ml. of active lung extract.

The cellulose powder was washed with 0·01 M NaCl solution repeatedly until the wash became clear and the cellulose packed in the column. The column was washed with an additional 100 ml. of 0·01 M NaCl solution, (pH 8·0).

Fifty ml. of lung-extract was put on the column and allowed to absorb. After absorption, the column was washed with 350–400 ml. of 0·01 M NaCl solution until the protein concentration in the eluate approached 50 γ/ml.

A double mixing flask apparatus (McGilvery, 1961) was set up as shown in Fig. 1 for eluting the material from the column. The 2 mixing flasks were connected to a reservoir which contained 1·0 M NaCl solution. The concentration of NaCl in the first mixing flask at the start of the experiment was 0·31 M (540 ml.) and the concentration of NaCl in the second mixing flask was 0·01 M (540 ml.). According to this method, a straight line increase in molarity of NaCl is obtained during the elution cycle. The concentration of NaCl at any given point can be readily calculated.

The eluates from the column were collected in 10–15 ml. aliquots in test tubes employing a time operated fraction collector.

Protein determination

The Folin-Ciocalteau method (Lowry, Roseburgh, Farr and Randall, 1951) and the spectrophotometric method (Ochoa, 1955) were used for determination of protein.

Lysozyme analysis

Suspensions of *Sarcina lutea* were obtained by washing the growth from 24 hr. slant Nutrient agar (Difco Laboratories, Detroit, Michigan) cultures with M/60 phosphate buffer in saline pH 6·5. The cell suspensions were adjusted with buffer-saline to give approximately 250 " nephelos " units in a Coleman nephelometer. Fresh cell suspensions were made daily for each run. Varying dilutions of the test samples were made in volumes of 1·0 ml. Five ml. of the standard suspension of *Sarcina lutea* was added to the dilutions of the test samples (1 ml.). The mixtures were incubated at 37° for 1 hr. in a water bath and nephelometric readings made.

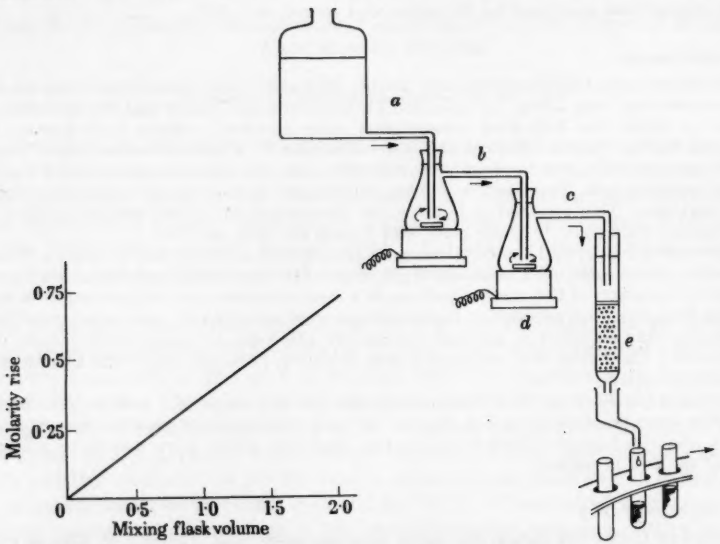

FIG. 1.—Double mixing flask apparatus for column chromatography. Abscissa of curve: mixing volume is the ratio of eluate volume to total mixing flask volumes. Ordinate of curve: rise in molarity of eluent above initial value 0·01 M NaCl.

a = Reservoir; *b* = 1st mixing flask; *c* = 2nd mixing flask; *d* = Magnetic stirrer; *e* = Cellulose column.

A standard control curve was determined for each run using varying known amounts of crystalline egg white lysozyme (Pentex, Inc., Kankakee, Illinois). The standard curve was plotted and the values for the unknown samples as determined from the standard curve were expressed in μg./ml.

RESULTS

Tuberculostatic activity and lysozyme content of rabbit lung extracts

Lung extracts made from rabbits which were vaccinated-challenged were tested against the BCG strain and *Myco. phlei*. Similar extracts prepared from the lungs of normal rabbits, of vaccinated rabbits (not challenged), and of challenged rabbits (not vaccinated) were tested in parallel. Each extract consisted of a pool of extracts made from 2 or more rabbits.

A typical protocol from such an experiment is shown in Table I. The vaccinated-challenged lung extracts showed the strongest activity against both BCG and *Myco. phlei*. *Myco. phlei* was more sensitive than BCG to the tuberculostatic

TABLE I.—*The Results of Bacteriostatic Tests with Lung Extracts*

Group	Strain	Dilution (final) of lung extract									
		1 : 6	1 : 12	1 : 24	1 : 48	1 : 96	1 : 192	1 : 384	1 : 768	1 : 1536	1 : 3072
Vaccinated-challenged	BCG	—	—	—	—	—	—	—	±	++	++
	Myco. phlei	—	—	—	—	—	—	—	—	—	++
Vaccinated	BCG	—	—	—	—	±	++	++	++	++	..
	Myco. phlei	—	—	—	—	—	—	—	+	++	..
Challenged	BCG	—	—	—	—	—	++	++	++	++	..
	Myco. phlei	—	—	—	—	—	—	+	++	++	..
Normal	BCG	—	—	—	±	++	++	++	++	++	++
	Myco. phlei	—	—	—	—	—	—	—	++	++	++

Myco. phlei incubated for 2 days. Control : ++
BCG incubated for 4 days. Control : ++

++ very good proliferation
+ good proliferation
± slight proliferation
— no growth

factor in lung extracts. Lung extracts from vaccinated animals (not challenged) and lung extracts from challenged animals (not vaccinated) were slightly more active than normal lung extracts. However, both were much less active than vaccinated-challenged lung extracts.

The lysozyme contents of the various lung extracts are presented in Table II. The lysozyme levels were approximately parallel to the levels of bacteriostatic activity of the lung extracts.

TABLE II.—*Lysozyme Content of Lung Extracts (of each group)*

Group	μg./g. of tissue.
Vaccinated-challenged	1350–1450
Vaccinated	450
Challenged	480
Normal	240–250

Column chromatography with Cellex CM cellulose

Typical results of column chromatography are shown in Fig. 2 and 3. Fig. 2 is from a pooled vaccinated-challenged lung extract and Fig. 3 is from a normal lung extract.

In these figures, the first protein fraction which was not absorbed to the column and which passed through the column is called " A " fraction. Fraction " A" contained no detectable lysozyme and was not noticeably altered by vaccination-challenge.

The second protein fraction " B " came down in 0·01–0·25 M of NaCl and probably contained several merging protein peaks. " B " fraction of normal lung extract was more extensive than the " B " fraction of vaccinated-challenged lung extract. However, this fraction did not possess lysozyme activity.

The third protein fraction " C " in Fig. 2 is much higher than " C " fraction of normal lung extract. In addition, lysozyme activity was much higher in " C " fraction of vaccinated-challenged lung extract than in " C " fraction of normal lung extract.

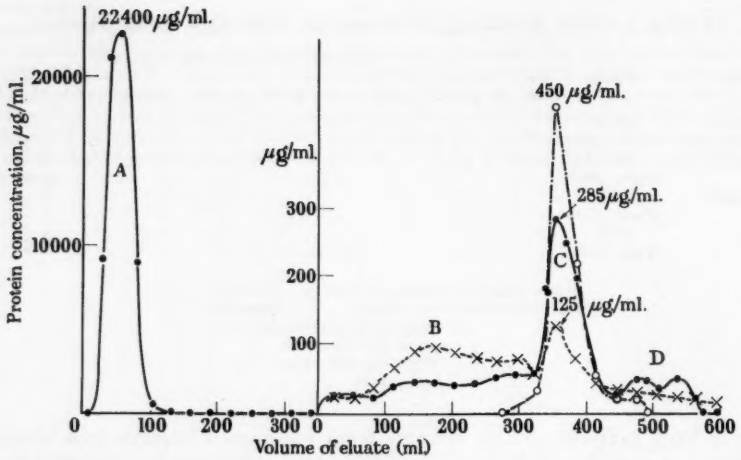

FIG. 2.—Column chromatography of vaccinated-challenged lung extract. Material: rabbit lung extract 50 ml., protein 30 mg./ml., lysozyme 450 μg./ml. Cellex-CM cation exchange cellulose: 5 g. Reservoir concentration 1·0 M NaCl. Mixing flask 1 starting concentration 0·31 M NaCl. Mixing flask 2 starting concentration 0·01 M NaCl. Mixing flask volumes 540 ml. each. pH 8·0.

○– – – – –○ Lysozyme
●————● Protein (Beckman)
×– – – – –× Protein (FCR)

For explanation of fractions A, B, C and D see text p. 141.

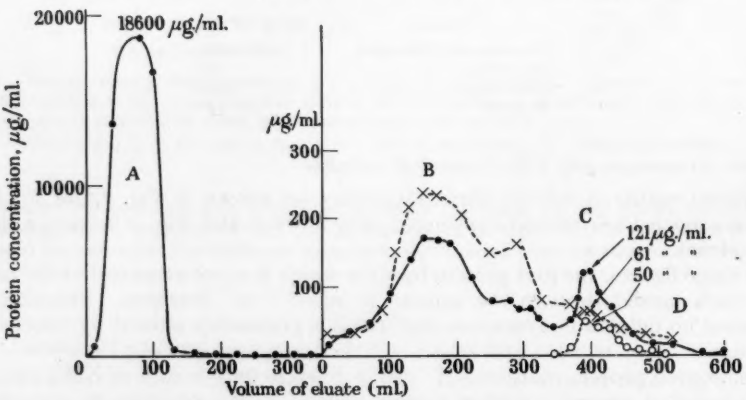

FIG. 3.—Column chromatography of normal lung extract. Material: rabbit lung extract 50 ml., protein 20 mg./ml., lysozyme 80 μg./ml. Cellex-CM cation exchange cellulose: 5 g. For data on reservoir concentration, mixing flask concentrations and volumes, see Fig. 2.

○– – – – –○ Lysozyme
●————● Protein (Beckman)
×– – – – –× Protein (FCR)

For explanation of fractions A, B, C and D see text p. 141.

The results of bacteriostatic tests on several points of the chromatographic curve from the lung extract of a vaccinated-challenged rabbit are shown in Table III.

TABLE III.—*The Results of Bacteriostatic Tests with Fractions of Vaccinated-Challenged Lung Extract*

Fraction	Against BCG Final dilution of Fraction.					Against *Myco. phlei* Final dilution of Fraction.						
	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64	1 : 128
A	++	++	++	++	++	++	++	++	++	++	++	++
B	++	++	++	++	++	++	++	++	++	++	++	++
B	++	++	++	++	++	++	++	++	++	++	++	++
B	++	++	++	++	++	++	++	++	++	++	++	++
C	−	−	−	+	++	−	−	−	−	−	−	±
D	−	+	++	++	++	++	++	++	++	++	++	++

Myco. phlei incubated for 2 days. Control : ++
BCG incubated for 4 days. Control : ++
See Table I for growth legend, ++, +, ± etc.

" A " and " B " fractions were devoid of detectable bacteriostatic activity.

" C " fraction (vaccinated-challenged) showed marked activity against both BCG and *Myco. phlei*. Fraction " D " showed slight activity and only against BCG. This activity suggests the presence of a second tuberculostatic factor other than lysozyme because *Myco. phlei.* was not inhibited. Lysozyme is more active against *Myco. phlei* than BCG.

DISCUSSION

The experimental approach described in this report is intended to reproduce the cellular milieu of the early stages of reinfection tuberculosis and offers a means for studying the intracellular and extracellular biochemical environment associated with this cellular response. It has been clearly shown that granulomatous lungs yield a relatively large amount of extractable tuberculo-inhibitory substances, whereas similar extracts from normal rabbit lungs are only weakly active.

Studies on the biochemical nature of the tuberculostatic factors has indicated that lysozyme appears to be the main tuberculostatic factor present in granulomatous lung extracts. A second factor representing approximately 20 per cent of the total tuberculostatic activity has been demonstrated by column chromatography. It possesses activity against BCG but not against *Myco. phlei*. Future studies will be directed towards the characterization of this second factor.

The cellular changes which occur in the granulomatous lungs involve cells of the mononuclear series almost exclusively. The demonstration of a high level of tuberculostatic activity of the extracts of granulomatous lungs would suggest that the cellular response seen in reinfection tuberculosis is indeed an important component of acquired immunity. In essence, the vaccinated animal is poised and ready to proliferate large numbers of immune macrophages upon second exposure to tubercle bacilli. In this regard, it is of interest that vaccinated rabbit lungs (not challenged) yield only slightly more tuberculostatic activity than normal lung extracts. These findings are compatible with the proposition that the granulomatous response represents an environment which is inimical to tubercle bacilli.

SUMMARY

A technique for producing a diffuse type of granulomatous response in the lungs of rabbits has been described. The data show that granulomatous lung extracts are 4–8 times more active in inhibiting the BCG strain of *Myco. bovis* than normal lung extracts. The dominant tuberculostatic factor has been isolated by column chromatography and this fraction contains a high level of lysozyme activity. A second less active tuberculostatic factor has been recognized in these extracts but awaits characterization.

The writers wish to thank Dr. R. W. McGilvery and Dr. W. A. Volk for much helpful advice concerned with column chromatography and also wish to thank Mrs. Sue Wilms for her technical assistance.

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THE TOXIC EFFECT ON RABBITS OF INFLUENZA VIRUS GIVEN INTRAVENOUSLY

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IT has been reported that intra-abdominal and intravenous injections of potent suspensions of influenza virus kill mice within 8–96 hr with necrotic lesions in the liver and haemorrhages in the alimentary tract (Henle and Henle, 1946). On the intravenous injection of Newcastle disease virus (NDV) into mice similar intestinal haemorrhages have been observed (Nakayama, 1958 ; Ogasawara and Yasue, 1959). According to the Henles (1946), these lesions were not the result of multiplication of the virus in these tissues, but due to the toxic effect which was inseparably associated with influenza virus particles. The nature of the viral toxin and the mechanism responsible for the production of the lesions are not fully understood.

In our studies it has been found that the intravenous injection of a concentrated suspension of influenza A virus (PR8) also causes toxic death of rabbits within 8–16 hr. Moreover, death occurred after injection of sonically vibrated virus suspensions which had lost most of their infectivity for chick embryos. Histological investgations revealed that the vascular system of the internal organs is generally and seriously injured. The present paper describes the experiments and the histological appearances of the virus-induced toxic reaction.

MATERIALS AND METHODS

Preparation of virus suspensions

The PR8 strain of influenza A virus was used. The virus was obtained by inoculation of 10-day-old chick embryos by the allantoic route with 0·25 ml. of infected allantoic fluid diluted 10^{-5}. After 48 hr. incubation at 37° the eggs were chilled at 4° overnight. The allantoic fluids were collected, clarified by preliminary centrifugation at 3,000 r.p.m. for 10 min., and then centrifuged at 44,000 **g** (23,000 r.p.m.) for 30 min. The pellets were resuspended in 0·01 M-phosphate buffered saline solution at pH 7·2 (PBS). The quantity of PBS used for resuspension was one-tenth of the original volume of allantoic fluid. The virus suspension was finally centrifuged at 3,000 r.p.m. for 15 min. to remove larger particulates. As controls, normal allantoic fluid and 20 per cent emulsions of normal embryos and chorioallantoic membranes from 12-day-old chick embryos were prepared. These were used after centrifugation at 3,000 r.p.m. for 10 min.

Preparations of inactive virus.—Virus suspensions were inactivated by heating for 30 min. at 56° or by ultraviolet irradiation at a distance of 30 cm. for 10 min. using a germicidal lamp (Mitsubishi Electric Co.).

Preparations of sonically vibrated virus.—The virus suspension was vibrated in a cup of Kubota's KMS–100 sonic vibrator (frequency, 10 kilocycles per second ; total power, 100 watts) for 1 hr. The cup was chilled with flowing ice-cold water throughout the vibration.

Haemagglutination (HA) titrations

HA titrations were usually carried out by the pattern method of Salk (1944), using 0·5 ml. of serial two-fold dilutions of the virus preparations made in PBS and 0·5 ml. of 0·5 per cent

suspensions of chicken red cells. The mixtures were incubated at room temperature and results recorded according to the patterns of the sedimented cells. The end-point was the highest dilution giving either complete or partial but definite agglutination. HA titres are expressed as values per ml.

Infectivity titrations

Serial tenfold dilutions of test materials were made in PBS and 0·25 ml. of each dilution were inoculated into the allantoic cavity of 10-day-old chick embryos. Four eggs were used for each dilution. After 72 hr. incubation at 37° allantoic fluids were harvested and tested for the presence of haemagglutinins. Titres were calculated in terms of ID_{50} per ml. according to the method of Reed and Muench (1938).

Animals

Rabbits weighing 2·0 to 2·5 kg. were used. The animals had been kept on our laboratory diet for at least 2 weeks before being used for the experiments. After intravenous injection of the virus suspension the animals were observed for 10 days. Death was immediately followed by necropsy and the internal organs were excised for virus titrations and pathological examinations.

Pathological examinations

The excised organs were carefully examined for gross pathological lesions and blocks of the organs were fixed in 10 per cent formalin for histological examination. Sections of the tissue blocks were stained by haematoxylin and eosin.

RESULTS

The lethal effect on rabbits of the intravenous injection of influenza virus

The results of intravenous injections of various doses of virus suspension and control materials into rabbits are summarized in Table I. As can be seen, death occurred within 8 to 16 hr. of intravenous injection of 2·5 ml./kg. body weight of

TABLE I.—*Lethal Effect of Intravenous Injections of Influenza Virus Suspension and Control Materials on Rabbits*

No. of rabbit	Material injected	HA titre	Log ID_{50}/ml. for chick embryos	Quantity injected (ml./kg. body weight)	Result of injection
	Virus suspension :				
1	Undiluted	40,960	10·4	2·5	Died, 8*
2	Diluted 1 : 2	20,480	10·1	2·5	,, 8
3	,, 1 : 2	20,480	10·1	2·5	,, 12
4	,, 1 : 4	10,240	9·8	2·5	,, 16
5	,, 1 : 4	10,240	9·8	2·5	,, 16
6	,, 1 : 8	5,120	9·5	2·5	Survived
7	,, 1 : 8	5,120	9·5	2·5	,,
8	Normal allantoic fluid			5·0	,,
9	chorioallantoic emulsion (20 per cent)			5·0	,,
10	chorioallantoic emulsion (20 per cent)			5·0	,,
11	chick embryo emulsion (20 per cent)			5·0	,,
12	chick embryo emulsion (20 per cent)			5·0	,,

* Died, 8 = Died within 8 hr.

virus suspensions having an HA titre higher than 10,240 ($10^{9.8}$ ID_{50}/ml.). On the other hand, the rabbits injected with virus suspensions having HA titres lower than 5,120 ($10^{9.5}$ ID_{50}/ml.) survived. Therefore, 10,240 × 2·5 HA units (10·2 ID_{50}) per kg. of body weight indicated an approximate minimal lethal dose of virus suspension. None of the control materials caused death of rabbits upon intravenous injection.

The lethal effect of virus suspensions with diminished infectivity for chick embryos

The results of intravenous injections of virus suspensions inactivated by heat or ultraviolet irradiation and of sonically vibrated virus suspensions are summarized in Table II. It can be seen that the lethal effect of influenza virus suspension was destroyed by heat (56° for 30 min.) and by ultraviolet irradiation (10 min.) but was rather strengthened by one hour's sonic vibration. On heating or ultraviolet irradiation the infectivity of virus suspensions for chick embryos was lost almost completely whereas the HA titre was only slightly decreased or unaltered. On sonic vibration most of the infectivity was lost but the HA titre was increased. Because an approximate minimal lethal dose of intact virus suspension by the intravenous route is $10^{10.2}$ ID_{50} per kg. of body weight as shown in Table I, the number of infective virus particles remaining in the sonically vibrated preparations could not cause toxic death of rabbits.

TABLE II.—*Lethal Effect on Rabbits of Intravenous Injections of Influenza Virus Suspensions with Diminished Infectivity*

No. of rabbit	Material injected	HA titre	Log ID_{50}/ml. for chick embryos	Quantity injected (ml./kg. body weight)	Result of injection
13	*Heated virus 56°, 30 min.	10,240	<1·5	2·5	Survived
14	†Ultraviolet-irradiated virus	40,960	<1·5	2·5	„
15	‡Sonically vibrated virus, undiluted	40,960	4·1	2·5	Died, 8§
16	‡Sonically vibrated virus, undiluted	40,960	4·1	2·5	„ 8
17	Diluted 1 : 2	20,480	3·8	2·5	„ 12
18	„ 1 : 2	20,480	3·8	2·5	„ 16
19	„ 1 : 4	10,240	3·5	2·5	„ 16
10	„ 1 : 4	10,240	3·5	2·5	„ 16
21	„ 1 : 8	5,120	3·2	2·5	„ 16
22	„ 1 : 8	5,120	3·2	2·5	Survived

* Before the treatment, 40,960 HA units/ml. and $10^{10.4}$ ID_{50}/ml.

† Irradiated by a germicidal lamp (Mitsubishi Electric Co.) at a distance of 30 cm. for 10 min. Before the treatment, 40,960 HA units/ml. and $10^{10.4}$ ID_{50}/ml.

‡ Sonically vibrated in a cup of Kubota's KMS–100 sonic vibrator (frequency, 10 kc./sec; total power, 100 watts) for 1 hr. Before the treatment, 20,480 HA units/ml. and $10^{10.1}$ ID_{50}/ml.

§ Died, 8 = Died within 8 hr.

Pathological appearance of rabbits intravenously injected with toxic suspensions of influenza virus

Two rabbits bled to death 3 hr. after intravenous injection of a lethal amount of virus suspension showed no pathological lesions. In all rabbits which died from intravenous injection of intact or sonically vibrated virus there were gross

pathological changes in the internal organs. The most conspicuous change was found in the lungs. They were markedly enlarged and bright red in colour, similar in appearance to the red consolidation of the lungs in mice inoculated intranasally with influenza virus. No obvious pleural and pericardial exudates were found. The right ventricle and atrium of the heart were filled with blood in spite of emptiness of the left ventricle. Intra-abdominal organs generally showed congestion, especially so the liver and spleen. These were usually enlarged and dark red in colour. However, mottling of the liver and haemorrhages in the alimentary tract, which are the usual findings at autopsy of mice injected intravenously with potent influenza A virus, were never found. The kidneys were slightly enlarged and reddish brown in colour; the adrenals showed no changes except slight congestion. The meninges and brain were moderately hyperaemic.

There was no difference grossly or microscopically between the autopsy findings in rabbits dead from the injection of intact virus and in those dead from the injection of sonically vibrated virus. Histological findings in the various organs were as follows (Figs. 1–5).

Lung: There were marked hyperaemia, haemorrhages and oedema but little infiltration with neutrophils, eosinophils and lymphocytes in the septa. The alveolar walls were markedly thickened. Many erythrocytes and much exudate were seen in alveolar spaces. There was no necrosis of the epithelium of the bronchi and bronchioles nor any peribronchial cell infiltration. The pleura was not affected. The appearances were those of acute pulmonary congestion and oedema.

Heart: Branches of coronary vessels were distended and filled with red blood cells. In the myocardium there were marked hyperaemia and numerous haemorrhages, and the spaces between heart muscle fibres were increased. Degeneration of the muscle fibres, however, did not occur. The endocardium and pericardium were not affected.

Liver: The normal architecture of the liver cells was well preserved, and degeneration or necrosis of the liver cells was not found. The small blood vessels and capillaries in the lobules and interlobular connective tissues were widely distended with blood cells, but infiltration with leucocytes was not found. The biliary tracts were not affected.

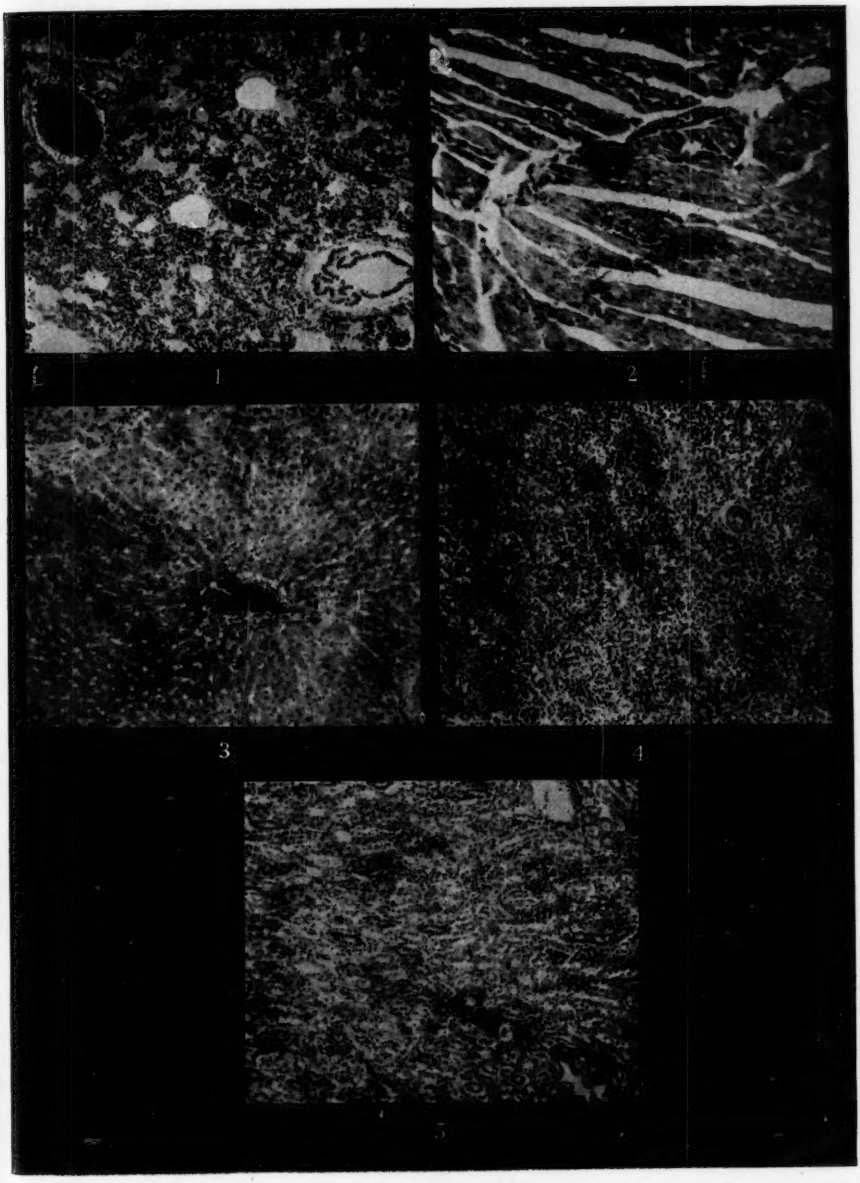
Intestines: No significant lesions were found except hyperaemia and congestion.

Spleen: The follicles were atrophic and contained few lymphocytes, and there was a moderate proliferation of large polygonal mononuclear cells. The sinusoids were widely distended with blood.

EXPLANATION OF PLATE

FIGS. 1–5 show the histological appearance of the various organs of a rabbit which died 16 hr. after the intravenous injection of 2·5 ml. per kg. of body weight of influenza virus suspension containing 10,240 HA units per ml. ($10^{9.8}$ ID_{50} per ml.). The tissue sections were stained by haematoxylin and eosin.

FIG. 1.—Lung. × 66.
FIG. 2.—Myocardium. × 130.
FIG. 3.—Liver. × 66.
FIG. 4.—Spleen. × 66.
FIG. 5.—Kidney. × 66.



Kato and Hara.

Kidneys : The glomeruli were generally and markedly enlarged owing to dilatation of the capillaries with red blood cells. In the interstitial tissues of the cortex and medulla, the arterioles, capillaries and small veins were markedly distended with blood. There were many haemorrhages. There was no degeneration of the epithelial cells of the tubules, the renal pelvis or calyces.

Brain : The small blood vessels and capillaries were markedly and diffusely distended with blood in the meninges and brain. There were also scattered haemorrhages into the brain. There was no indication that the lesions were limited to special portions of the brain. The nerve cells did not show any alteration.

In summary, the histological changes in the rabbits were generalized severe dilatation of the blood capillaries and small blood vessels in the internal organs, accompanied by capillary haemorrhages of varying degree depending on the organ. Other inflammatory signs, such as an infiltration by leucocytes, were unusual in all of the organs except the spleen. The most severe lesions of the capillaries were found in the lungs.

The fate of influenza virus injected intravenously into rabbits

Immediately upon death the various organs of intravenously injected rabbits were removed and emulsified in PBS to form 10 per cent suspensions. The blood was obtained from the right ventricle of the heart. In addition 2 rabbits, injected with a lethal dose of virus, were killed by exsanguination 3 hr. after the injection and the organs were likewise suspended in PBS and served as controls. The blood was obtained by heart puncture before killing. The concentration of virus in the blood and organs was determined by infectivity titrations in chick embryos. Haemagglutinin could not be detected in any of the preparations. The results of virus titrations in chick embryos are given in Table III. Three hours after the injection virtually all virus had disappeared from the blood. Virus was recovered nearly exclusively from the lungs, much smaller amounts were present in the kidneys, and little or none was present in the brain, spleen, liver and small intestine. The total amount of virus detectable in the organs by infectivity titrations 3 hr. after the injection amounted to only a few per cent of the injected virus. By the time of death, 16 hr. after the injection of the virus, the titre in the lungs and kidneys had decreased and the total amount of virus detectable in the organs tested was less than one per cent of the injected virus.

DISCUSSION

The experiments reported in this paper establish the fact that the intravenous injection of concentrated suspensions of influenza A virus (PR8) causes toxic death of rabbits within 8 to 16 hr. Histological investigation revealed generalized damage to the capillaries and related changes in the internal organs. From the histological findings it can be assumed that the direct cause of death of the rabbits is acute pulmonary congestion and oedema. It has been reported that the intravenous injection of potent suspensions of influenza A virus causes death of mice with mottling in the liver and haemorrhages in the intestinal tract (Henle and Henle, 1946). Such lesions were not found in rabbits.

Three hours after the intravenous injection virus was recovered nearly exclusively from the lungs. In other organs and blood much less or no virus could be

TABLE III.—*Location of Virus in the Organs and Blood of Rabbits Injected Intravenously with Influenza Virus*

No. of rabbit	Time after the injection	Material	Result of virus titration‡ Log ID_{50}/g.	Log ID_{50}/organ
24	3 hr., killed	Brain	2·1	3·4
		Lungs	7·4	8·9
		Liver	<1·5	<3·3
		Spleen	2·5	3·1
		Intestine	<1·5	<3·5
		Kidneys	4·5	5·7
		Blood*	<1·5	—
25	3 hr., killed	Brain	2·0	3·3
		Lungs	7·7	9·2
		Liver	<1·5	<3·3
		Spleen	2·7	3·3
		Intestine	<1·5	<3·5
		Kidneys	3·8	5·1
		Blood*	<1·5	—
4	16 hr., died	Brain	1·9	3·1
		Lungs	6·8	8·5
		Liver	<1·5	<3·5
		Spleen	<1·5	<2·5
		Intestine	<1·5	<3·5
		Kidneys	4·1	5·3
		Blood†	<1·5	—
5	16 hr., died	Brain	1·7	2·9
		Lungs	6·2	7·7
		Liver	<1·5	<3·5
		Spleen	<1·5	<2·5
		Intestine	<1·5	<3·5
		Kidneys	3·8	5·0
		Blood†	<1·5	—

* Blood obtained by heart puncture.

† Blood in the right ventricle of the heart. Rabbits were injected intravenously with 2·5 ml. per kg. body weight of virus suspension (10,240 HA units/ml. $10^{9.8}$ ID_{50}/ml.).

‡ Infectivity titrations in chick embryos.

detected. The site of adsorption of virus in the lungs is unknown. Until death there was no evidence of virus multiplication in the lungs and other organs as determined by infectivity and haemagglutination titrations. Histologically, no necrosis of the epithelial cells of the bronchi and bronchioles and no peribronchial cell infiltration were observed in contrast with the usual finding in the lungs of mice infected intranasally with influenza virus (Straub, 1937). The lesions and deaths produced by the intravenous injection of influenza virus in rabbits are therefore not the result of virus multiplication.

The total amount of virus detectable in the tested organs by infectivity titrations 3 hr. after the injection was only a few per cent of the injected virus. The exact mechanism of the loss of virus is poorly understood. Mims (1959) reported that considerable amounts of the viral antigen were present in large numbers of littoral cells in the liver shortly after the intravenous injection of influenza virus. He maintains that the phagocytic cells in the reticuloendothelial system are largely responsible for the rapid clearance of the virus from the blood.

Influenza virus suspensions, inactivated by heat or ultraviolet irradiation, lost their lethal effect on rabbits and infectivity for chick embryos, whereas their HA titres were well maintained. On the other hand, rather increased toxicity was demonstrated in the virus suspension which had been sonically vibrated for 1 hr. and had lost most of its infectivity. The HA titre of the sonically vibrated suspension was somewhat increased. It was reported that sonic vibration by a less powerful oscillator than the one used here, did not affect the infectivity of influenza virus in 2 hr. treatment (Wiener, Henle and Henle, 1946 ; Kirber and Henle, 1950 ; Lief and Henle, 1956). In the most recent studies in our laboratory, however, it has been confirmed by electron microscopy and ultracentrifugation that the influenza virus particles are disintegrated by intense sonic vibration (Kato, unpublished).

From the findings with inactivated virus suspensions it can be assumed that the virus-induced toxicity depends on a component which is not responsible for haemagglutination and infectivity. Henle and Henle (1946) have already described in a paper on the toxicity of influenza virus in mice how upon exposure of influenza virus preparations to heat, formalin, or ultraviolet irradiation the infectivity for chick embryos was lost at a faster rate than the toxic property. But the separation of the toxic component from the virus particles has not hitherto been accomplished. The disintegration of the virus particles by sonic vibration may be a useful method for the separation of the toxic component from the virus particles. Ether-treatment has been found to disintegrate influenza virus particles (Hoyle, 1952 ; Hoyle, Reed and Astbury, 1953 ; Schäffer and Zillig, 1954). This treatment, however, destroys completely the toxic effect of the virus particles. Experiments to separate the toxic component from influenza virus particles are now in progress.

The exact mechanism responsible for the toxic death and lesion production in rabbits injected intravenously with a large dose of influenza virus is not fully understood. From the pathological changes, however, two ideas may be suggested as regards the primary site affected by the viral toxin. Either the toxin acts directly upon the vascular endothelium and seriously alters the vascular permeability, or the vasomotor nervous system is the primary target and the damage to the blood vessels is a secondary change. The data presented in this paper provide no definite evidence to support either of these suggestions. Wilcox and associates (1958), working on the corneal toxicity induced by the intraocular injection of either NDV or influenza A virus, have indicated that the viruses are capable of acting more or less directly upon at least one type of endothelial cell in such a way as to destroy the functional integrity of the membrane which these cells compose. Kempf and Chang (1949), and Chang and Kempf (1950) reported that male albino rats injected intravenously with influenza A or B viruses showed a lowering of arterial blood pressure due to a toxic property of the viruses, and that this hypotensive factor was intimately associated with the virus particle. According to their results, the hypotensive factor acted on the heart but produced no detectable changes either in the calibre of, or the rate of flow through the mesenteric blood bessels. Ogasawara and associates (1958, 1959) have found that administration of chlorpromazine or tetraethylammonium bromide before intravenous or intranasal inoculation of NDV protects mice from the toxic lesions to a considerable degree. Since these drugs also block impulses in the autonomic nervous system, this result suggests the participation of this system in the patho-

genesis of NDV-induced toxic lesions. Clearly, further studies will be required to determine the exact mechanism responsible for the virus-induced toxic reactions.

SUMMARY

The intravenous injection of concentrated suspensions of influenza A virus (PR8) causes toxic death of rabbits within 8 to 16 hr. Histological characteristics of the rabbits were generalized damage to the blood capillaries and related changes in the lungs, myocardium, liver, spleen, kidneys, intestines and brain. The most conspicuous change was found in the lungs. It is assumed that the direct cause of death of the rabbits is acute pulmonary congestion and oedema.

A large part of the virus disappeared shortly after injection. The remainder was recovered nearly exclusively from the lungs. In the other organs much less or no virus could be detected. Until death there was no evidence of virus multiplication in the lungs and other organs as determined by infecivity and haemagglutination titrations.

Toxic death of rabbits was also caused by intravenous injection of virus suspensions which were sonically vibrated for 1 hr. and had lost most of their infectivity. Histological appearances in these rabbits were identical with those in rabbits injected with intact virus. Heated or ultraviolet-irradiated virus suspensions had lost their infectivity and toxicity, whereas the haemagglutinin titres were well maintained. From these facts it can be assumed that the virus-induced toxicity depends upon a toxic component which is not responsible for infectivity and haemagglutination.

We wish to express our gratitude to Prof. K. Ogasawara for his encouragement throughout the present studies.

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COMPOSITION AND SOURCE OF SECRETION FROM LYMPHOID AGGREGATIONS IN THE RABBIT GUT

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ALTHOUGH it has long been known that ligation of a rabbit's caecal appendage led to its distention and subsequent perforation (Behan, 1921), it was not until the work of Dennis, Buirge, Varco and Wangensteen in 1940 that the comparative physiology of the caecal appendage was investigated. Dennis found that of 25 representative birds and mammals studied, only man, the chimpanzee and the rabbit had a caecal appendage possessing the power of secretion. In these 3 animals secretion at a pressure as high as arterial pressure was observed and perforation invariably followed luminal obstruction.

A common feature of the appendices in these species is the preponderance of lymphoid tissue. The rabbit, which has the larger proportion of lymphoid tissue, secretes the largest volume. It is tempting therefore to associate appendiceal secretion with lymphoid tissue. However, in certain species, such as the tiger, where there is no true appendix, the function of the caecum is poised in the direction of absorption, despite the presence of considerable lymphoid tissue in the mucosa (Dennis *et al.*, 1940).

The significance of the power of secretion in the genesis of acute appendicitis in man was recognized by Van Zwalenberg (1904) and fully investigated by Wangensteen and Dennis (1939). The problem of finding the cells responsible for secretion then became of practical importance.

The present study was designed to estimate the volume and composition of the secretion from the parts of the rabbit gut, where aggregations of lymphoid tissue are found, and to try to discover the importance of the lymph follicles in the production of this secretion.

MATERIALS AND METHODS

Throughout these experiments, New Zealand white rabbits of both sexes were used. Adults were anaesthetized with pentobarbital sodium and the newborn with ether.

Collection of secretions was made by cannulation of the gut with a 5 mm. outside diameter glass cannula. The lumen was obstructed distally after gentle lavage with saline solution. A balloon was attached to the cannula and the whole returned to the coelomic cavity. A second laparotomy was performed 24 hr. later for collection of the secretion. Balloons were used of sufficient capacity to prevent a rise in intraluminal tension and were chemically clean.

Histological examination of the alimentary tract lymphoid tissue was made in adult, newborn and foetal rabbits using standard haematoxy in and eosin stain. Mucoid material was identified in goblet cells in sections stained by the methyl green-pyronin method of Scudder (1944).

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RESULTS

Distribution of alimentary lymphoid tissue and morphology of the appendix

The distribution of the major aggregations of lymphoid tissue is shown in Fig. 1 (Bensley, 1948). In these areas, there is a striking similarity of structure (Fig. 2). The other small collections of lymphoid tissue found in the wall of the gut are not associated with this characteristic structure (Fig. 3). A diagrammatic representation of the structure of the appendix wall is shown in Fig. 3. Two distinct types of epithelium are seen, one covering the villi and the other covering the heads of the lymphoid follicles. The latter has been designated

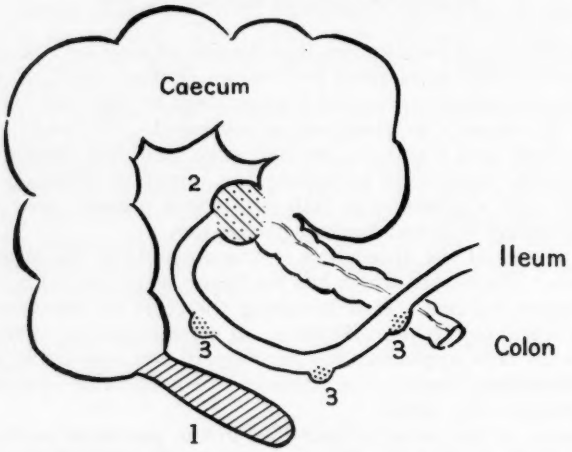

FIG. 1.—Alimentary lymphoid tissue. (1) Appendix, (2) Saccus rotundus, (3) Agminate patches.

" capital epithelium " in preference to the term " lympho-epithelium " (Crabb and Kelsall, 1940). The villous epithelium is uniformly composed of tall columnar cells almost all containing mucin. The capital epithelium is less regular but consists of a single layer of epithelial cells, which are freely infiltrated by lymphocytes. The capital epithelium does not produce mucin (Fig. 4).

Volume of secretion from various segments of the gut

The average size of the organ and volume of secretion estimated from the findings in 12 adult rabbits (Table I) shows that secretion occurs in those areas where lymphoid tissue is found. For technical reasons, not all the collections were made in the same animal, but a total of 6 collections from each site was made except for the colon, where there were only 2 collections.

The apparent inconsistency in the volume of secretions from the ileum are due to the initial outpouring of fluid into an obstructed small bowel, even when this part of the bowel is normally poised in the direction of absorption. The longer loop was less affected by obstruction than the shorter.

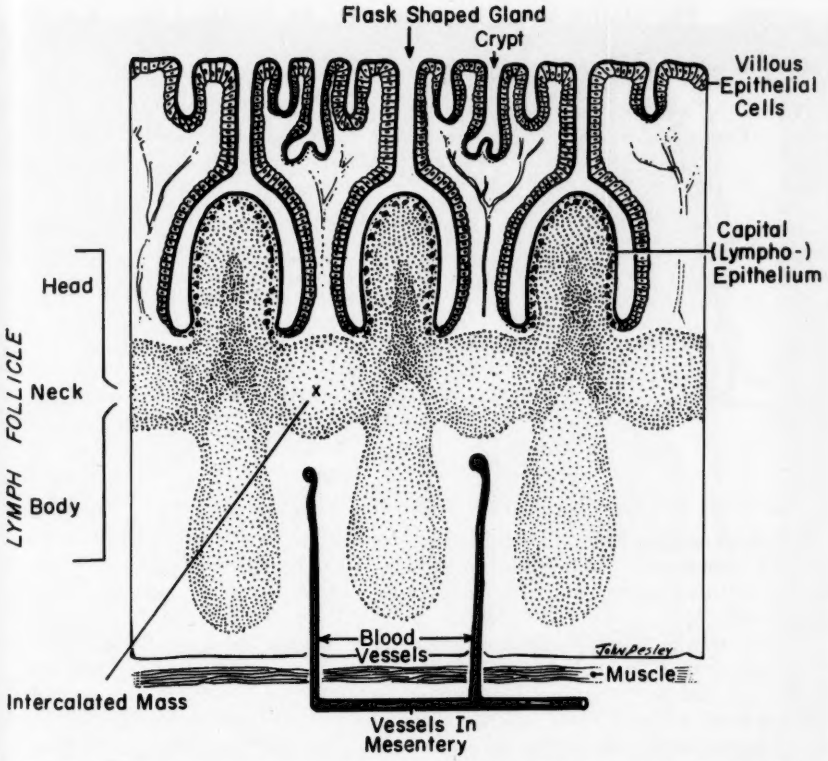

FIG. 3.—The term "Lympho-epithelium" of Crabb and Kelsall has been abandoned in favour of the term "Capital epithelium".

TABLE I.—*Mean Values from Six Measurements in Adult (2·5 kg.) Rabbits*

Organ	Weight (g.)	Length (cm.)	24-hr. secretion (ml.)
Appendix	10	8	40
Saccus rotundus	7·4	3	22
Ileum with lymphoid	1·5	3	6
Ileum without lymphoid	1·2	3	4
	5	16	2
Colon	4·5	10	None

The composition of the secretion

Table II shows the range of results obtained from the secretions of 12 appendices and 6 sacci rotundi. There was no significant difference in the secretions from the 2 sites.

The secretion from the gut with the major aggregations of lymphoid tissue is seen to be a serous alkaline juice rich in bicarbonate ions and low in calcium and

phosphates. The relative serum levels are CO_2, 14–25 mEq/l Ca, 5·6–8 mEq/l and phosphates 5·9 mg./100 ml. (Spector, 1958). The function of this secretion is being investigated and will be reported upon later.

TABLE II.—*Composition of Secretion from Rabbit Appendix and Saccus Rotundus*

	Range	
pH	8·1–9·4	
S.G.	1·001–1·006	
*Viscosity	1·110–1·180	centipoise
†Osmolarity	251–287	mileosmoles
Sodium	132–150	mEq./l.
Potassium	6·7–1·9	,,
Calcium	0·35–0·95	,,
Bicarbonate	64–82	,,
Chloride	68–85	,,
Phosphates	0·4–1·6	mg./100 ml.

* Hess' method. Corrected to 20°.
† Fiske Osmometer.

The role of the lymphoid tissue in the production of the secretion

The appendix of the newborn rabbit does not contain any lymphoid tissue, although occasional lymphocytes may be seen at the bases of the villi. By the 3rd day of life, aggregations of these lymphocytes form the earliest lymphoid follicles (Fig. 5).

Twelve newborn rabbits had a laparotomy within the first 12 hr. of life (6 within the first 4 hr.) The base of the appendix was gently obstructed by a silk ligature, taking care to avoid damage to the blood vessels in the mesentery. In 2 newborn, a comparable length of caecum was obstructed between ligatures. After closure of the abdomen, the rabbits rapidly recovered and were returned to the mother. The selection of a docile mother and careful handling throughout the latter part of pregnancy was necessary in order to ensure her acceptance of her newborn offspring following their operation. Before sufficient satisfactory mothers were found, some rabbits had destroyed their young at birth or after operation.

Two of the newborn rabbits died within 12 hr. of operation. In both of these there was microscopic evidence of distention of the appendix. Macroscopic distention was present in all the 6 killed 12–24 hr. after operation (Fig. 6).

In the comparable lengths of obstructed caecum, no distention was present, macroscopically or microscopically, 24 hr. after operation.

Perforation had occurred in 3 rabbits killed 48 hr. after operation, and in 1

EXPLANATION OF PLATES.

FIG. 2.—A, Appendix. B, Saccus rotundus, × 35. C, Agminate patch, × 35.

FIG. 4.—The epithelium covering the head of the lymph follicle has no mucin-containing cells but is infiltrated with lymphocytes. The epithelium lining the villi (Rt.) is almost entirely composed of goblet cells containing mucin. Stained with methyl green pyronin, × 185.

FIG. 5.—Appendix of newborn rabbit. Note the absence of lymphoid tissue at the base of villi. × 185.

FIG. 6.—Newborn rabbit appendix after 24 hr. obstruction, showing distal distention.

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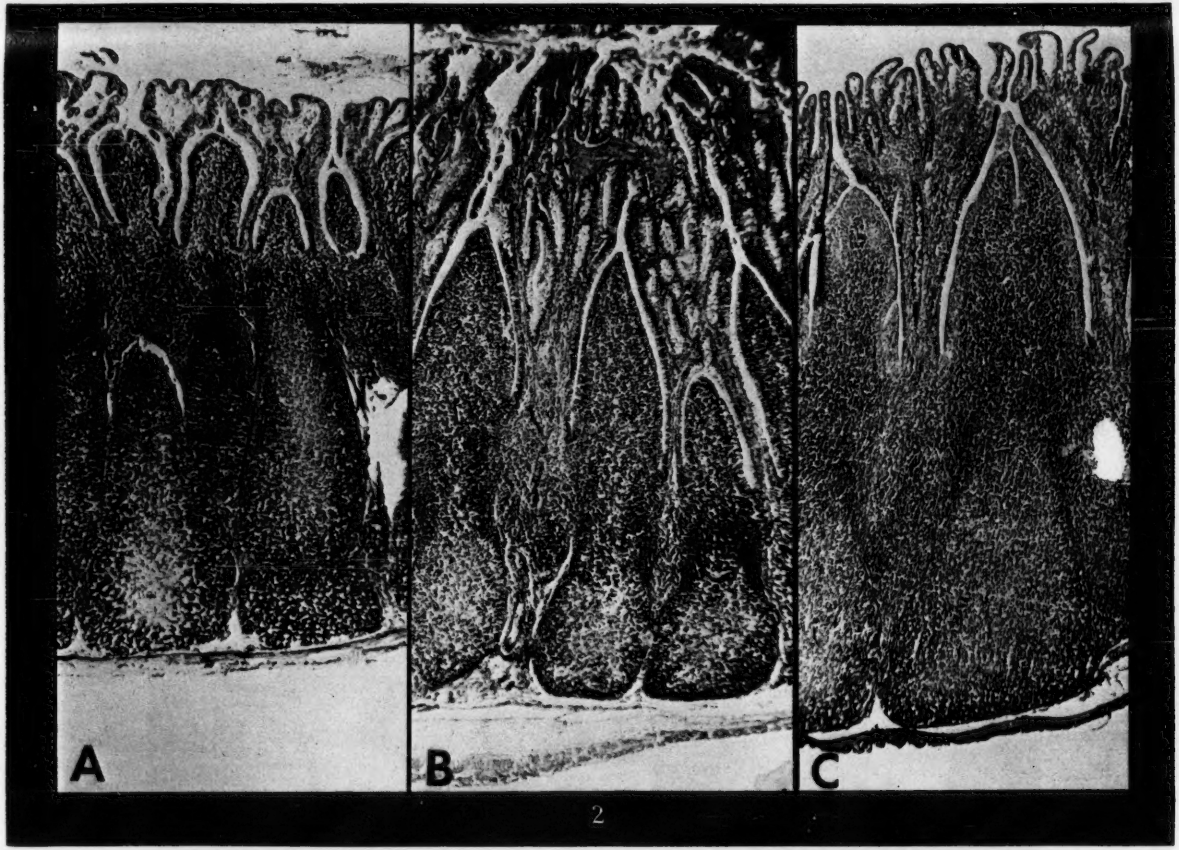

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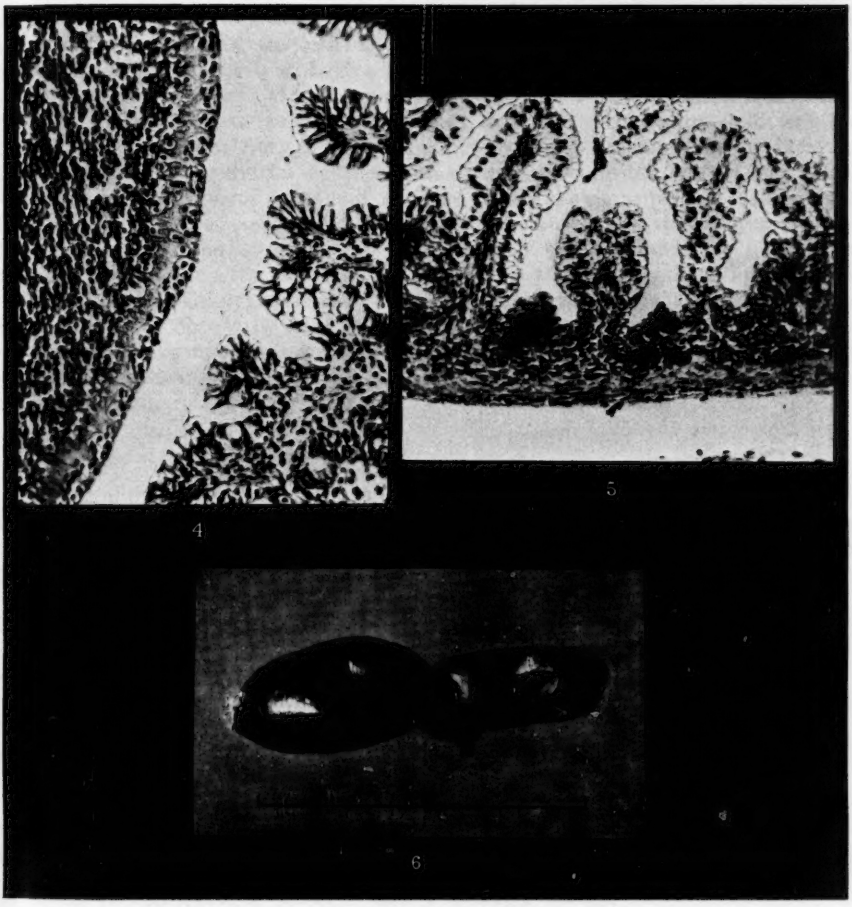

animal killed at the 7th day of life, a spontaneous fistula had developed between the distal appendix and the ileum.

DISCUSSION

The secretion of a large volume of alkaline juice from parts of the rabbit's ileum and caecum is associated with those areas where there is abundant lymphoid tissue. The search for the cells responsible for the secretion has led to the attempt to separate the lymphoid and epithelial elements of the tissues. Perry, Loken and Wangensteen (1959) investigated the effect of irradiation and corticoids in an attempt to suppress lymphoid activity. Irradiation sufficient to cause suppression of activity of the lymphoid tissue, also damaged the epithelium. Corticoids did not produce a significant alteration in secretion, neither was there a marked suppression of lymphoid activity. The rabbit appendix is balanced in the favour of secretion whereas the caecum is balanced in favour of absorption. In the newborn, the appendix secretes whereas the caecum does not. The essential difference in the histological structure of these 2 organs at birth is the presence of epithelial aggregations at the base of the villi in the appendix ; these are destined to form the capital epithelium. Secretion occurs prior to the development of lymphoid tissue and is therefore presumably independent of the lymphoid tissue, and probably is the function of specialized epithelial cells at the base of the villi.

SUMMARY

In the alimentary tract of the rabbit, the appendix, the saccus rotundus and the agminate patches of the ileum all secrete into the lumen of the gut. The secretions of the appendix and saccus rotundus are identical and are found to be serous, alkaline and rich in bicarbonate ions.

Secretion appears to be associated with areas of the gut containing aggregations of lymphoid tissue. The power of secretion, however, is independent of the presence of lymphoid tissue, as secretion is shown to occur in newborn rabbit appendix prior to the development of lymphoid tissue.

The cells responsible for secretion are probably those epithelial cells in close relation to the lymphoid tissue.

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ATTEMPTED INDUCTION OF IMMUNOLOGICAL TOLERANCE IN RABBITS USING LIVING *TREPONEMA PALLIDUM*

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THE phenomenon of immunological tolerance, postulated by Burnet and Fenner (1949) and proved by the now classic experiments of Billingham, Brent and Medawar (1953), has been extensively investigated from many different aspects in recent years. While Traub's (1938) observations concerning lymphocytic choriomeningitis virus in mice, formed a cornerstone of Burnet and Fenner's (1949) hypothesis, inquiry into tolerance to microbial antigens has been characterized by many failures and few successes. Unsuccessful experiments with non-living bacterial antigens by Smith and Bridges (1958), Wager and Hautala (1957), Cohn (1957), Nossal (1957) and others, have presumably prompted Burnet (1959) to state that it has proved almost impossible to produce tolerance against bacterial antigens. Wicher and Rogalowa (1960) using killed *Treponema pallidum* in neonatal rabbits were unable to show suppression of reagin formation upon subsequent challenge with living *Trep. pallidum*. Following intratesticular challenge, however, a difference in the nature of the lesion was noted in the pretreated rabbits as compared with the control rabbits. Employing killed *Salmonella pullorum* in fowls and *Trichomonas foetus* in very young calves, Buxton (1954) and Kerr and Robertson (1954) found a significant degree of inhibition of antibody formation following respectively embryonic and neonatal pretreatment with the above antigens.

Experiments designed to induce tolerance with living pathogenic microbes usually had fatal consequences (Burnet, Stone and Edney, 1950, Buxton, 1954, Nossal, 1957). Attempts to interpret these findings in relation to tolerance have not been made. Smith and Bridges (1958) and Weiss (1958) used living *Mycobacterium tuberculosis* BCG in neonatal rabbits and in embryonic guinea pigs respectively, and adopted the suppression of the tuberculin skin reaction as their criterion of tolerance; they were unable to demonstrate any deviation from the adult response. They did not, however, report on the clinical course of the disease in these particular experimental circumstances. Nossal (1957) reported no suppression of antibody formation in mice 4 weeks old, which had previous intra-uterine injections of minute doses of live influenza virus. Neither the clinical course of the surviving mice, nor the fate of the inoculated virus was described.

Traub (1938) produced tolerance by infecting embryonic mice with lymphocytic choriomeningitis virus, and his work has been confirmed by Hotchin (1958).

In most experiments in which deliberate attempts to induce tolerance have been made, a single manifestation of the specific immune response such as suppression of antibody formation or hypersensitivity phenomena of the delayed type was chosen as a criterion, in terms of which the results were interpreted. In

view of the conflicting observations, it was decided to examine further the possibility of inducing tolerance to a living microbial antigen. Rabbits were chosen as experimental animals with *Trep. pallidum* as the antigen because it is usually non-fatal in rabbits, can be withdrawn at will by treatment with penicillin, and the presence of antibodies can be determined in several ways.

MATERIALS AND METHODS

Rabbits.—Adult male and female rabbits, aged 12–18 months, were used. Their sera, tested prior to use, showed no evidence of reagin or immobilisin activity. During the course of these experiments, which were carried out over a period of 2½ yr, 6 litters were bred.

Antigen.—Nichol's strain of *Trep. pallidum* was obtained from early rabbit orchitides and the dosage employed, unless otherwise stated, was 5×10^6 treponemes. In parallel with the inoculation of each group of experimental animals, a normal adult control rabbit was inoculated intratesticularly, giving syphilitic orchitis in all cases.

The presence of treponemes was demonstrated by dark-field microscopy or biologically by intratesticular injection of suspected material. These rabbits were clinically, bacteriologically and serologically observed for 3–6 months.

Serological tests.—Rabbits were bled sterilely from the ear. The presence of reagin was detected by the Kolmer (initial dilution 1 : 2·5) and V.D.R.L. tests; immobilisin by the Treponema Pallidum Immobilization test (Bokkenheuser and Richardson, 1959).

Histology.—Except for the rabbits that died from neglect within the first week of life, all dead rabbits were submitted to necropsy. Sections were taken from all the internal viscera and were stained with haematoxylin and eosin and by the Warthin-Starry method.

Temperature measurements.—Rectal temperatures of a 100 Himalayan rabbits were measured with an " Electric Universal Thermometer Type TE 3 " (Ellab Elektrolaboratoriet).

RESULTS

Infection of pregnant rabbit does

Trep. pallidum was inoculated subcutaneously into two does, 4 days and 6 days after mating, respectively. Clinical syphilis was not observed. Serological examinations were carried out at intervals over the following 4 months. Reagin was evident at 6 and 8 weeks, while immobilisin was found to be present at 6 and 16 weeks respectively (Table I).

TABLE I.—*Infection of Pregnant Rabbit Does*

Rabbit doe	Infection	Response		
		Clinical	Antibodies	
			Reagin	Immobilisin
I	4 days after mating	Nil	+ve 6w	+ve 6w
II	6 days after mating	Nil	+ve 8w	+ve 16w

w = weeks after infection

Search for evidence of transplacental infection

Of the 16 rabbits born to the infected does, 5 were from litter I and 11 from litter II. There were no stillbirths. Two rabbits from litter I and 5 rabbits from litter II died from neglect within the first 5 days.

S1 and S2 (Table II), represent animals killed at 20 days and 1 day respectively. In each case evidence of syphilitic infection was absent by darkfield examination of the blood, by biological tests using homogenized liver, by serological investigations for reagin and immobilisin and by histological examination.

At no stage, during the subsequent 12–14 week period of observation did the survivors show any evidence of syphilitic infection, when examined clinically, serologically, by dark-field examination of the blood or by biological test using pooled blood. The same holds true for the survivor which was observed for 36 weeks.

Failure to induce immunological tolerance in the offspring of syphilitic does

Despite the failure to demonstrate transplacental infection it was thought that antigenic material might still be crossing the placental barrier to cause tolerance. Two of the offspring (Table III) were accordingly challenged intratesticularly at 13 weeks and one was kept as a control. The rest were challenged subcutaneously at 18 weeks. The clinical and serological responses were indistinguishable from those of normal adult rabbits. A week later an orchitis developed in each of the 2 rabbits challenged intratesticularly. Tests for reagin and immobilisin became positive in all the experimental rabbits, except two which remained serologically negative during the observation period of 18 weeks, but developed antibodies 14 weeks after intradermal rechallenge.

Attempts to induce immunological tolerance by infecting neonatal rabbits

Having failed to produce tolerance by infecting the pregnant doe, neonatal rabbits were inoculated subcutaneously with *Trep. pallidum* within 5 hr. of birth. The clinical course of the 10 inoculated rabbits, derived from 4 different litters showed a consistent pattern; they developed normally up to the 5–7th week, after which stunted growth was evident. This was followed by a progressive decline in nutritional state and a purulent discharge from the eyes with lesions spreading to adjacent tissues.

Of the 7 rabbits examined serologically, all developed reagin and 5 were found to have immobilisin between 7 and 14 weeks. The remaining 3 rabbits were not examined serologically owing to technical difficulties. All the rabbits became cachectic and died within 16 weeks. Organs examined histologically included brain, liver, lungs, spleen, kidneys, aorta, heart and suprarenals. None of the changes demonstrated were diagnostic of syphilis but the perivascular round cell infiltration present in most brains examined, was felt to be consistent with a diagnosis of syphilis. Other organs including the lungs, kidneys and spleen showed in most cases non-specific round cell infiltration. Only in one case were spirochaetes demonstrated in the brain and spleen. The heart and aorta were normal or showed non-specific changes.

Attempted induction of tolerance in neonatally infected rabbits by interrupting the infection

The final series of experiments was designed to determine whether the hypersusceptible state persisted in the neonatally infected rabbits when the disease was terminated with penicillin and *Trep. pallidum* reintroduced at a later stage. Three groups of rabbits were obtained from 4 different litters. The group

TABLE II.—*The Search for Evidence of Transplacental Infection*

Rabbit doe	Infection of pregnant doe	Newborn rabbits: Total	Still-births	Neonatal deaths	Survivors	Time of examination	Response: Dark-field	Transfer	Antibodies: Reagin	Immobilisin	Histology
I	28 days before birth	5	Nil	2 (neglect)	S1	3w	−ve	−ve	−ve	−ve	−ve
					1 and 2	12w	−ve	−ve	−ve	−ve	
II	26 days before birth	11	Nil	5 (neglect)	S2	1 day	−ve	−ve	−ve	−ve	−ve
					3–7	14w	−ve	−ve	−ve	−ve	

w = age of rabbits in weeks
S1 and S2 = killed rabbits

TABLE III.—*The Response to Living* Trep. Pallidum *of Rabbits Born to Infected Does*

	Rabbit number	Antibodies prior to challenge: Reagin	Immobilisin	Challenge: Age of rabbits	Route of infection	Response: Clinical	Antibodies: Reagin	Immobilisin	Rechallenge: Age of rabbits	Route of infection	Response: Clinical	Antibodies: Reagin	Immobilisin
Experimental rabbits	1 and 2	−ve 12w	−ve 12w	13w	it	Orchitis 14w	+ve 18w	+ve 18w	..	..	..	..	..
	3	−ve 14w	−ve 14w	18w	sc	Nil	+ve 31w	+ve 36w	..	..	..	..	..
	4 and 5	−ve 14w	−ve 14w	18w	sc	Nil	−ve 36w	−ve 36w	46w	id	Nil	+ve 60w	+ve 60w
Control rabbit	6	−ve 14w	−ve 14w	—	—	Nil	−ve 36w	−ve 36w	46w	id	Nil	+ve 60w	+ve 60w

w = age of rabbits in weeks
it = intratesticular
id = intradermal
sc = subcutaneous

" P.A.M. treated syphilitic rabbits " (Table IV), comprised 8 animals which were inoculated with *Trep. pallidum* within 5 hr. of birth and 5 weeks later treated with penicillin in 2 per cent aluminium monostearate. Three to 5 consecutive daily, intramuscular injections of 30,000–60,000 units were given. The " P.A.M. treated control rabbit " was not infected but when 5 weeks old, was nevertheless given 4 consecutive injections of P.A.M. (30,000 units) daily by the intramuscular route. The last group " untreated control rabbits " was neither infected nor given penicillin and consisted of 2 rabbits. Most infected rabbits showed weak reagin activity about the 12th week, which in all but one case (rabbit 9) disappeared before the 18th week. The latter lost its reagin by the 30th week. Immobilisin was undetectable in all rabbits. Between 22–33 weeks, the rabbits were challenged with *Trep. pallidum*, the first 2 groups intradermally, and the untreated control group subcutaneously. After 3–4 weeks following challenge, chancres developed at the site of inoculation in 3 out of 8 experimental animals and in the " P.A.M. treated control rabbit ". All except 2 experimental animals (9 and 10) and one untreated control rabbit (18) responded by the production of immobilisin and reagin by the 28th–62nd week. Evidence of spirochaetaemia was not obtained from rabbits 9 and 10 by biological testing of the blood.

Rabbits 9, 10 and 18 were rechallenged intradermally at 52–62 weeks, 9 and 10 receiving 30–40 $\times$ 10^6 treponemes by the combined intravenous and intradermal routes. Chancres developed at the site of intradermal inoculation in rabbits 9 and 10 and reagin and immobilisin between 64 and 68 weeks in all 3 animals. All the rabbits were observed for a further period of 7 months and none showed any evidence of clinical disease. The chancres at the site of inoculation healed within 3–4 weeks. The fulminating disease of the neonatal rabbits was not reproduced.

Three rabbits not shown in Table IV were discarded in the course of the investigation. One served as an uninoculated, untreated control, another was originally in the " P.A.M. treated control rabbit " group and developed reagin and immobilisin at 21 weeks. It is not possible to explain this finding satisfactorily as this rabbit never received an injection of *Trep. pallidum*. The third rabbit died of intercurrent bronchopneumonia at 16 weeks.

DISCUSSION

Failure to produce congenital syphilis in rabbits born from syphilitic mothers confirms the work of Pautrizel, Mayer, Rivasseau-Coutant and Szersnovicz (1957). These authors state that the nature of the protective element extant in rabbit foetuses is unknown. Weber (1953) showed that the survival of *Trep. pallidum in vitro* was progressively shorter as the temperature was increased from 20° to 40° and that treponemes do not survive above 40°. The temperature at which treponemes ordinarily multiply *in vivo* is in the range of 30–38°, probably with an optimum between 35° and 37° (Turner and Hollander, 1957).

Lesions fail to develop in the rabbit testis if after inoculation the testis is pushed up into the warmer abdominal cavity for a few hours (Bessemans, 1930). Turner and Hollander (1957) have repeated this experiment and concluded that the optimum temperature range for the multiplication *in vivo* of treponemes is less than the internal body temperature of the rabbit. Over the period of a year we measured the rectal temperatures of a 100 Himalayan rabbits, of which 70 per

TABLE IV.—*Neonatally Infected Rabbits Treated with Penicillin, and Reinfected as Adults*

	Rabbit number	Antibodies prior to challenge: Reagin	Antibodies prior to challenge: Immobilisin	Challenge: Age of rabbit	Challenge: Route of infection	Response: Clinical	Response: Antibodies: Reagin	Response: Antibodies: Immobilisin	Transfer	Rechallenge: Age of rabbit	Rechallenge: Route of infection	Response: Clinical	Response: Antibodies: Reagin	Response: Antibodies: Immobilisin
P.A.M.* treated syphilitic rabbits	8	−ve	−ve	25w	id	Nil	+ve 36w	+ve 36w	..	..	..	..	..	..
	9	Kolmer 20u.	−ve	25w	id	Nil	−ve 48w	−ve 48w	−ve 52w	52w	id and iv	Chancre 57w	+ve 64w	+ve 64w
	10	−ve	−ve	32w	id	Nil	−ve 53w	−ve 53w	−ve 58w	58w	id and iv	Chancre 60w	+ve 68w	+ve 68w
	11–15	−ve	−ve	33w	id	Chancre 3/5	+ve 41w	+ve 41w	..	..	..	..	..	..
P.A.M.* treated control rabbit	16	−ve	−ve	33w	id	Chancre 36w	+ve 44w	+ve 44w	..	..	..	..	..	..
Untreated control rabbits	17	−ve	−ve	22w	sc	Nil	+ve 28w	+ve 62w	..	..	..	..	..	..
	18	−ve	−ve	22w	sc	Nil	−ve 62w	−ve 62w	..	66w	id	Nil	+ve 66w	+ve 66w

Abbreviations: see previous Table
iv = intravenous

* Penicillin in 2 per cent aluminium monostearate

cent registered temperatures between 38·1° and 38·7°, slightly lower than those recorded by Bessemans (1930). The intra-uterine temperature can be expected to be as high, if not higher than the rectal temperature and certainly not lower than the intra-abdominal temperature. It seems probable therefore that treponemes are unable to multiply or survive in this environment, and that no protective mechanisms other than the environmental temperature is required to eliminate the spirochaetal infection from the rabbit foetuses. It may reasonably be assumed that if an infection had been established, death in utero would have resulted. The absence of congenital syphilis does not exclude the possibility that antigenic components of *Trep. pallidum* might cross the placental barrier. Should they pass over it might be expected that their presence in the embryo may be revealed by antibody formation, or if present in sufficient quantities, and for a long enough period, by tolerance. Neither reagin nor immobilisin were detected in the baby rabbits born from syphilitic does and upon later inoculation with *Trep. pallidum* they behaved as normal adults. However, we have not excluded the possibility of prenatal exposure to the antigen as a transient tolerance might have been demonstrated by earlier postnatal challenge.

Infection of rabbits at birth resulted in a rapidly progressive disease, with fatal termination between the 8th and 16th week, although antibody formation proceeded normally and overwhelming numbers of treponemes were not found in the tissues. The death of these rabbits might possibly be due to a metabolic disturbance to which the mature adult rabbit is not susceptible. The administration of penicillin at 4–5 weeks cured the rabbits, thus indicating that the presence of the organisms beyond this stage is essential to produce a fatal infection. Upon reinfection with living *Trep. pallidum* 22–24 weeks later, the resulting response was no different from that of a normal adult animal infected for the first time.

If tolerance is defined as the failure to form antibodies, these experimental rabbits were not tolerant. However, Hildeman and Medawar (1959) and Hasek and Hort (1960) have shown that the development of circulating antibodies does not necessarily lead to the rejection of tolerated skin grafts. By analogy, if tolerance can be assumed to exist in our rabbits irrespective of the presence of antibodies, the treponemes should be able to multiply freely. Yet, we have no evidence of a massive accumulation of spirochaetes in the tissues. Considered in this light, it cannot be averred that tolerance was present.

As a result of our findings several experiments have been formulated. Firstly, to determine at which age young rabbits infected with syphilis switch from the neonatal to the adult response. Secondly, to interrupt the disease of neonatally infected rabbits at about this age and to test the effect of immediate reinfection.

SUMMARY

Rabbit does infected with *Trep. pallidum* 4 and 6 days after mating were apparently unable to transmit the infection transplacentally to their offspring. Infection was not suggested serologically, histologically, by dark-field examination or by biological testing of suspected material. Evidence indicates that this failure may be due to the temperature in the pregnant rabbit uterus.

Based on the absence of congenital syphilis in rabbits born from syphilitic does, the absence of antibody formation and the absence of tolerance, we have no evidence that *Trep. pallidum* or its antigenic components responsible for

immobilisin or reagin formation, are capable of crossing the placental barrier. The antibody response of these animals and their resistance to *Trep. pallidum* when challenged as adults remained indistinguishable from the response in normal adults.

Infection of rabbits at birth while causing no inhibition of antibody formation resulted in a rapid, progressive and fatal disease, the majority of deaths occurring within 10 weeks. The response was almost identical in rabbits derived from four different litters.

Rabbits infected with living *Trep. pallidum* at birth, cured with penicillin 4 weeks later, and challenged at 6 months of age behaved clinically and serologically as normal adults. Susceptibility of the baby rabbits to fatal infection by *Trep. pallidum* is not a permanent characteristic once the course of the infection has been interrupted.

Tolerance to *Trep. pallidum* was not demonstrated in any of our experiments.

We have pleasure in thanking Mr. N. J. Richardson for technical assistance, Miss G. McNab for performing the reagin tests, Drs. C. Abrahams and J. C. E. Kaufman for histological opinion, Dr. A. Zoutendyk for the temperature observations on the rabbits, Drs. D. J. Goldstein and C. G. Anderson for valuable criticism and the Director of this Institute Dr. J. H. S. Gear for facilities granted.

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THE EFFECT OF DIETARY FATS AND SYNTHETIC GLYCERIDES ON THE PRODUCTION OF ATHEROSCLEROSIS AND THROMBOSIS IN THE RAT

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THROMBOSIS and atherosclerosis can be produced independently in the rat by feeding a diet containing 2 different fats (Gresham and Howard, 1960). Thus rats fed with butter cholesterol, cholic acid and thiouracil produce thrombosis. If butter is replaced by arachis oil, no thrombosis is seen but lesions are produced, closely resembling those of human atherosclerosis. Butter differs from arachis oil in having a high content of saturated fatty acids but a low content of linoleic acid. The present work is concerned with effects of feeding, natural and synthetic fats with widely different fatty acid compositions.

MATERIALS AND METHODS

Source of fats.—Butter and beef fat were the normal retailed products. Hydrogenated arachis oil (m.p. 34°) was kindly supplied by the British Oil and Cake Mills Ltd., Erith, Kent. Arachis oil was obtained from British Houses, Poole, Dorset; maize oil from the Clyde Oil Extraction Co., Glasgow.

Synthetic glycerides.—Glyceryl mono-oleate distearate was prepared according to the method of Wheeler, Riemenschneider and Smith (1940). Oleic acid (33 g., British Drug Houses), stearic acid (666 g., British Drug Houses), glycerol (102 g.) and *p*-toluene sulphonic acid (10 g.) were heated under oxygen free nitrogen at 120–130° for 5 hr. The mixture on cooling was added to 3 l. petroleum ether (b.p. 40–60°) and washed with 70 per cent ethanol containing 4 g. sodium hydroxide, followed by 4 further washes with 70 per cent ethanol only. After washing 3 times with water, the petroleum ether layer was dried over sodium sulphate and the solvent removed at 100° under nitrogen. Final traces were removed by vacuum pump. This method gives a mixture of symmetrical and unsymmetrical triglycerides and no attempt was made to purify the product further.

The other glycerides were prepared in a similar manner using the following quantities: glyceryl dioleate mono stearate:—oleic acid (666 g.) stearic acid (333 g.); glyceryl trioleate:—1 kg. oleic acid; glyceryl trilinoleate:—1 kg. linoleic acid.

Linoleic acid and methyl linoleate were prepared by standard methods (Biochemical Preparations, 1955).

Animals and experimental design.—The animals and basic diet used (Table I) were similar to those described previously (Gresham and Howard, 1960). Groups of 5–10 rats were fed the basic diet containing different fats as described below. Table II shows the different fats used together with their fatty acid composition (Groups 1–5). Three groups of rats were fed mixtures, *viz.* Group 6: butter and arachis oil mixed (1 : 1); Group 7: butter containing 8 per cent (W/W) methyl linoleate (thus producing a total linoleic acid content approximating that of arachis oil); Group 8 was fed arachis oil for 8 weeks and then changed to butter. Synthetic fats given were glyceryl trilinoleate (Group 9), glyceryl mono-oleate distearate (Group 10), glyceryl dioleate mono stearate (Group 11), and glyceryl trioleate (Group 12).

Pathological examination.—Animals were allowed to continued on the diets until death occurred. Necropsy and treatment of tissues was as described previously (Gresham and Howard, 1960).

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TABLE I.—*Basic Diet*

Active ingredients*	g.
Fat	400
Cholesterol	50
Cholic acid	20
Thiouracil	3

* Other ingredients were: sucrose 169 g., casein 200 g., choline chloride 10 g., salts 40 g., cellophane flaked film 100 g., magnesium oxide 5 g., inositol 2 g., thiamine hydrochloride 16 mg., pyridoxine hydrochloride 16 mg., folic acid 10 mg., calcium pantothenate 40 mg., biotin 0·6 mg., B_{12} 0·05 mg., nicotinamide 0·2 g.; a weekly supplement to each rat comprising 1000 i.u. vitamin A and 40 i.u. vitamin D supplied as one drop halibut liver oil, 2 mg. α-tocopheryl acetate and 0·05 mg. 2-methyl-1 : 4 naphthoquinone.

TABLE II.—*Different Fats Used and their Composition** (*per cent mole*)

Group	Fat	Fatty acids		
		Saturated	Oleic acid	Linoleic acid
1	Butter	65	30	5
2	Beef fat	55	43	2
3	Hydrogenated arachis oil	25	73	2
4	Arachis oil	20	55	25
5	Maize oil	15	25	60

* Hilditch (1956)

RESULTS

Different fats

Results are given in Table III.

Animals given beef fat and hydrogenated arachis oil died first after 4–8 weeks. Those given butter, arachis and maize oil lived from 10–14 weeks. Butter, beef fat and hydrogenated arachis oil produced thrombosis of the cardiac chambers, aorta and coronary arteries, myocardial infarction (Fig. 1) and large clusters of lipid filled macrophages at the apex of the heart, on the endocardial surface of the mitral valve and on the aortic intima. These 3 fats have a somewhat similar composition consisting chiefly of glycerides containing saturated fatty acids linked to oleic acid.

TABLE III.—*Distribution of Lesions in Rats given Different Fats*

Group	Fat in diet	No. of animals	Range of survival time (weeks)	Atherosclerosis	Lipid-filled Macrophages	Thrombosis	Myocardial infarction
1	Butter	25	11–17	0	18	13	6
2	Beef fat	7	4–8	0	6	2	1
3	Hydrogenated arachis oil	6	4–10	0	2	3	1
4	Arachis oil	8	10–18	7	1	0	0
5	Maize oil	7	11–22	3	1	2	0

Those fed arachis oil showed no thrombosis or infarction and few lipid filled macrophages. In contrast atherosclerotic plaques were produced in the proximal aorta (Fig. 2*a*) and coronary arteries. These plaques consisted chiefly of fibrous

material and contained lipid at the base. Plaque formation appears to be associated with fats containing a high proportion of linoleic acid since rats given hydrogenated arachis oil (containing a negligible amount of linoleic acid) did not show atherosclerosis. Butter and beef fat also have low linoleic acid contents.

Likewise, maize oil (60 per cent linoleic acid) produced atherosclerotic plaques but these contained much more lipid than with arachis oil (Fig. 2*b*). An unexpected result was the production of intra cardiac thrombosis and thrombi related to the aortic atherosclerotic plaques. Animals given arachis oil did not develop thrombosis.

Mixture of fats

The results are shown in Table IV.

Butter and arachis oil mixed in equal amounts produced the sum of lesions given by each independently: atherosclerosis in addition to thrombosis and infarction. As was expected a similar result was seen when rats were fed arachis oil for 8 weeks and then changed to butter. Addition of methyl linoleate to butter, produced atherosclerosis in addition to thrombosis and infarction which occurred with butter alone. This result would support the view that linoleic acid is essential for the production of atherosclerotic plaques.

TABLE IV.—*Distribution of Lesions in Rats given Mixtures of Fats*

Group	Mixture of fats	No. of animals	Range of survival time (weeks)	Atherosclerosis	Lipid-filled macrophages	Thrombosis	Myocardial infarction
6	Butter and arachis oil (1 : 1)	7	9–24	3	1	1	2
7	Butter and methyl linoleate (8 per cent)	4	7–20	3	1	2	2
8	Arachis oil for 8 weeks followed by butter	7	12–18	4	1	5	1

Synthetic glycerides

The results are shown in Table V.

Linoleic acid alone, when given as glyceryl trilinoleate, did not however produce atherosclerosis. This result would suggest that its effect depends on the presence of saturated fatty acids such as occur in small proportion in maize and arachis oil. Two animals showed an accumulation of lipid filled macrophages but no other lesions were seen.

Two glycerides (group 10 and 11), which simulate those occurring in butter and beef fat, produced thrombosis and aggregates of lipid filled macrophages.

EXPLANATION OF PLATE.

FIG. 1.—Heart of rat given hydrogenated arachis oil showing thrombosis in the aorta and coronary artery and a septal infarct. (H. and E. × 33.)

FIG. 2*a*.—Aorta of rat given arachis oil showing fibrous plaque with small deposition of lipid in base. (H. and E. × 78.)

FIG. 2*b*.—Aorta of rat, given maize oil showing plaque with abundant lipid. (H. and E. × 82.)

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2a 2b

Gresham and Howard.

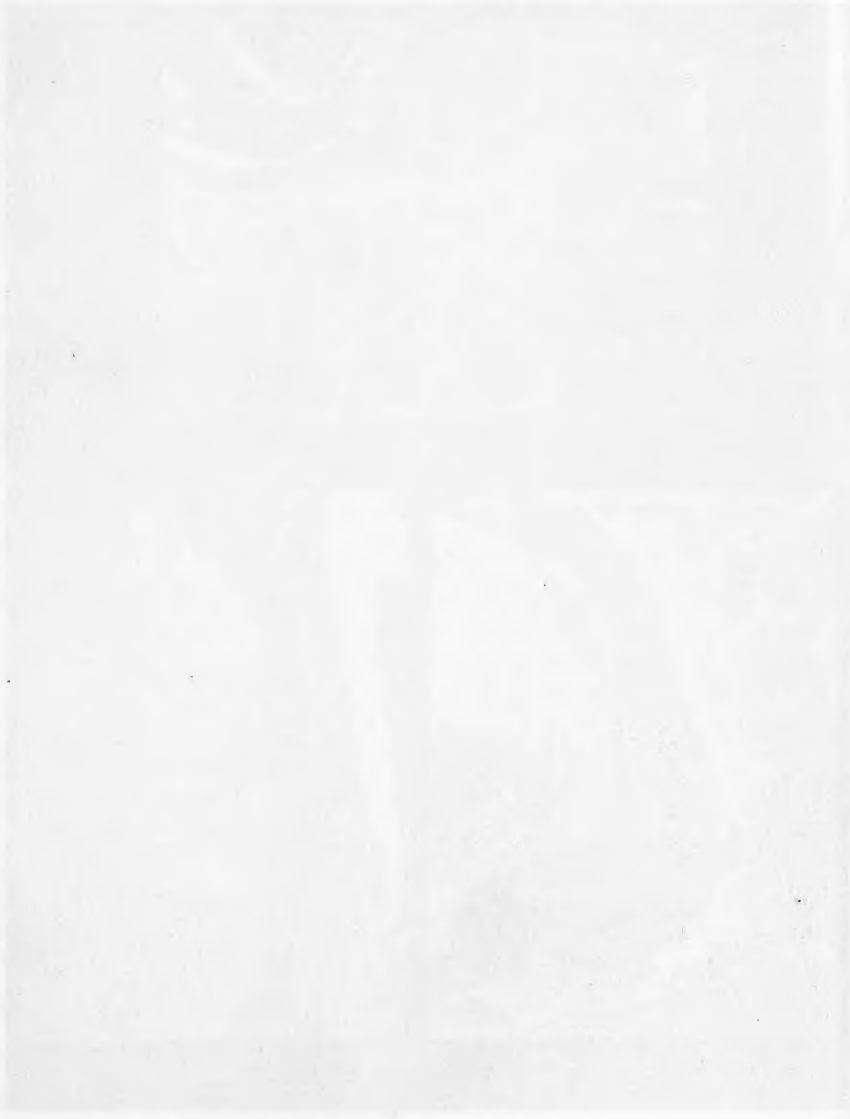

TABLE V.—*Distribution of Lesions in Rats Given Synthetic Glycerides*

Group	Glyceride	No. of animals	Range of survival time (weeks)	Atherosclerosis	Lipid filled macrophages	Thrombosis	Myocardial infarction
9	Trilinoleate	5	8–9	0	2	0	0
10	Mono-oleate-distearate	10	2–4	0	8	6	0
11	Di-oleate mono-stearate	5	4–5	0	4	2	0
12	Tri-oleate	8	5 survived 16 weeks	0	0	0	0

More thrombosis was seen with the glyceride containing the greater amount of saturated fatty acid (group 10). Myocardial infarction was not seen but it should be noted that these animals died after only a short period, on the diet. The essential component for thrombosis is a saturated fatty acid since no lesions were observed if oleic acid was given alone as glyceryl trioleate (group 12).

DISCUSSION

Our previous results (Gresham and Howard, 1960) showed that the production of atherosclerosis or thrombosis and myocardial infarction is dependent on the type of fat in the diet. Thus arachis oil together with agents which produce hypercholesterolaemia, cholesterol, cholic acid and thiouracil (Thomas and Hartroft, 1959) produces atherosclerosis whereas replacement of arachis oil by butter produces thrombosis and infarction. In the present experiments the nature of these differences has been clarified. Animal fats such as butter or beef fat which contain chiefly saturated fatty acid linked with oleic acid produced thrombosis in the cardiac chambers, aorta and coronary arteries causing myocardial infarction. Two glycerides resembling the components of animal fats, namely glyceryl mono-oleate distearate and dioleate mono-stearate were found to produce thrombosis. The essential component was shown to be a saturated fatty acid since oleic acid alone as the trioleate was ineffective. Moreover thrombosis was greater in the glyceride containing the greater amount of saturated fatty acid, namely, glyceryl mono-oleate distearate.

Vegetable oils such as arachis and maize, which contain a high content of linoleic acid produced atherosclerosis closely resembling that seen in man. Linoleic acid appears to be important in plaque formation since no plaques were seen after hydrogenation of arachis oil, a procedure which removes linoleic acid. Moreover the addition of methyl linoleate to butter produces atherosclerosis in addition to thrombosis. It should be noted however that glyceryl trilinoleate alone is without effect and indicates the necessity for the presence also of saturated fatty acids. Current investigations are concerned with the effect of glycerides containing linoleic linked to saturated fatty acids.

Our results are in accord with the recent work of Böttcher, Woodford, Romeny-Wachter, Ter Haar, Houte and Bodsma van Gent (1960) who showed conclusively that human atherosclerotic plaques have a high content of linoleic acid which increases with the severity of the disease. In contrast the results offer no support

for the hypothesis of Sinclair (1956) who believes that atherosclerosis is caused by a relative deficiency of essential fatty acids such as linoleic.

An unexpected finding was that maize oil could also cause thrombosis in addition to atherosclerotic. This oil contains little saturated fatty acids and of these only a negligible amount is linked to oleic (Hilditch, 1956). Arachis oil does not produce thrombosis but although the fatty acid content of the two oils are similar the manner in which they are linked in the glycerides is markedly different (Hilditch, 1956). A further investigation into the factors involved in this difference would seem to be of value and is being undertaken.

SUMMARY

Rats were given a diet containing 40 per cent fat, 5 per cent cholesterol, 2 per cent cholic acid and 0·3 per cent thiouracil, and the effects of natural and synthetic fats on thrombosis and atherosclerosis investigated. Butter, beef fat, hydrogenated arachis oil and synthetic glycerides containing stearic and oleic acids produced thrombosis. Arachis oil produced atherosclerosis without thrombosis. Mixtures of butter and arachis oil, butter supplemented with 8 per cent methyl linoleate, and maize oil produced both lesions. None was observed with glyceryl trioleate and trilinoleate. It was concluded that saturated fatty acids are thrombogenic and that linoleic acid in conjunction with saturated fatty acids is atherogenic.

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THE TECHNIQUE AND INTERPRETATION OF TESTS FOR LEUCOCIDIN WITH SPECIAL REFERENCE TO THE VALUE OF ETHYLENE DIAMINE TETRA-ACETIC ACID (EDTA)

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IN 1959 the authors described a method of determining the leucocidal effects of many substances, mostly antiseptics and antibiotics. This was based on earlier work of Achard and Ramond (1912) with reference to oscillation of the granules of the polymorphonuclear leucocyte. Both in the course of this work and in its continuation during the last 2 yr. in which observations have been directed more to the leucocidal effects of bacterial toxins, we have been conscious of defects in the technique which make these observations unduly tedious and sometimes difficult of interpretation, especially if the toxin under examination has haemolytic and leucolytic activity such as Gladstone and Van Heyningen (1957) observed in preparations of staphylococcal toxin containing δ haemolysin.

The method used to obtain the leucocyte suspensions required for the observations previously described (McLeod and McLeod, 1959) depended on the use of heparin as anticoagulant and ox fibrinogen to accelerate the separation of the blood cells, as recommended by Minor and Burnett (1948). Exposure to hypotonic solutions of NaCl containing low concentrations of the vital stain neutral red had been used following the technique of Achard and Ramond (1912) to distinguish lightly-stained cells with oscillating granules as living from strongly-stained or colourless cells with stationary granules as dead.

These observations were unduly time-consuming and tedious because of the tendency to the development of coagula in leucocyte suspensions so obtained. This increased the longer the leucocytes were kept after separation and had the effect of eliminating a large proportion of the leucocytes from the test since when enmeshed in these coagula they were superimposed one on another so that their outlines were obscured and it was impossible to know how far they had been protected in the depths of the coagulum from the effects of the substance of which the leucocidal effect was under observation. Consequently the count had to be made on the rather scanty free-lying cells and this required much time. Further, where the total time needed for a count on a considerable number of tubes took 2–3 hr., it was often observed that the number of active cells in the control tubes without toxic substance added had fallen away considerably towards the end of the period of observation. Although, therefore, the method of Minor and Burnett (1948) had been found to give better yields of leucocytes from blood than any other tried up till that time, it was in some respects defective for our purpose. It was necessary, therefore, to find methods which: (i) gave at least as good a yield of leucocytes as Minor and Burnett's, and prevented any

considerable formation of clots or clumps in the leucocyte suspensions prepared: (ii) to prepare and observe suspensions of leucocytes in dilutions of toxin, etc., in such a way that control tubes showed no damage to the leucocytes over a period of 1 hr.

In more recent work on the separation of blood cells new procedures have been adopted; the use of EDTA, the chelating agent, as an anticoagulant and the sedimentation of the red blood corpuscles after mixing whole blood with anticoagulant and concentrated solution of dextran so as to leave the leucocytes and platelets in the supernatant plasma, also the practice of preparing all glassware used with silicone. The method we have adopted is based on the work of McKinney, Martin, Rundles and Green (1953); Brecher, Wilbur and Cronkite (1953); Zucker and Borelli (1958); Bond, Fliedner, Cronkite, Rubini, Brecher and Schork (1959), and Gold and Cole (1960).

MATERIALS AND METHODS

Method of separating leucocytes and observing leucocidal effects

All operations in obtaining leucocyte suspensions are carried out with vessels and pipettes treated with silicone.

A volume of 10 ml. of freshly-drawn blood is run into a mixture of 1 ml. of 10 per cent dextran in 5 per cent dextrose solution (Benger's " Dextraven ") and 1 ml. of a 1 per cent solution of the disodium salt of ethylene diamine tetra-acetic acid (EDTA) in 1·4 per cent NaCl. This mixture is left standing at room temperature for 30 min. The plasma which has separated is then centrifuged and is found to yield a sediment of approximately 0·1 ml. of blood, leucocytes and platelets in which the leucocytes are about as numerous as the red blood corpuscles.

The sediment, which is found to disperse easily, is taken up in 2 ml. of 20 per cent plasma in 0·85 per cent NaCl solution and is found on microscopic examination to contain a large proportion of isolated cells and some small loose clumps of leucocytes, red blood corpuscles and platelets in which the outline of the leucocytes is well defined.

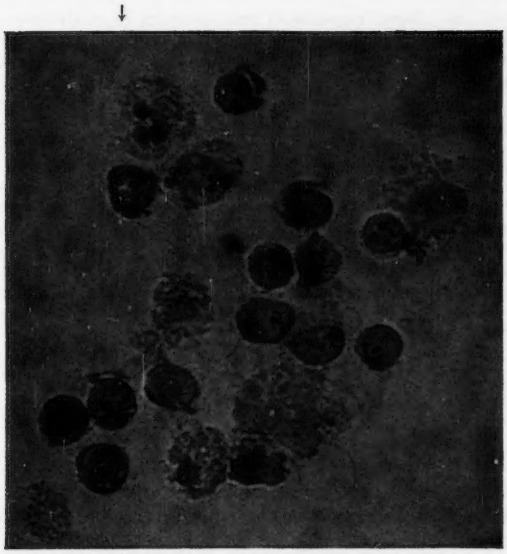
In observing the lethal effect of antiseptics, toxins etc., on the leucocytes, dilutions of the substance under observation are prepared in Hanks' solution containing 0·1 per cent of EDTA and neutralized afresh, since a mixture of Hanks' (pH 7·3) and 1 per cent EDTA (pH 7·3) rapidly develops an acid reaction (about pH 5).

Each tube receives 2 drops of leucocyte suspension and after incubation for 1 hr. or longer the cells are spun down, the supernatants are sucked off and each sediment is taken up in a 1–2000 solution of methylene blue in 0·3 per cent NaCl solution. A loopful of this mixture is mounted between slide and coverslip and examined under 1/12 oil-immersion objective. Cells of the granular series are observed for oscillation of the granules and staining.

EXPLANATION OF PLATE.

FIG. 1.—Wet preparation of dead leucocytes suspended in dilute methylene blue in 0·3 per cent sodium chloride solution. Leucocytes after subjection to the action of haemolytic and leucocidal toxin. The majority of the cells present are probably lymphocytes. There are, however, a number of granular cells in which the granules stain blue and also the nucleus: the best example is the cell nearest the arrow.

FIG. 2.—This is the control preparation. Wet preparation of living leucocytes suspended in dilute methylene blue in 0·3 per cent sodium chloride solution. The granules in the cells which have unstained or very slightly stained nuclei were in an active state of oscillation. Red blood corpuscles are relatively intact. Granules in the polymorphs which were in active movement appear as dark particles unstained, and there is very little nuclear staining in these cells. Such a cell spread out in amoeboid form is to the left of the lowest red blood corpuscle. This preparation is unduly dark because only a brief exposure was possible in order to avoid blurring from rapid oscillation of the granules.



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Cells showing active oscillation and colourless or very faintly coloured nuclei are considered living cells, those with frank staining and some persisting oscillation are considered moribund and those without oscillation of granules are considered dead. In the great majority of observations the latter are deeply stained. Lymphocytes and mononuclears are not counted. Where the colourless and oscillating cells are reduced to less than 50 per cent of those in the control the substance is considered to be leucocidal at the concentration tested. The appearances described are shown in Fig. 1 and 2.

In dealing with leucolytic toxin such as that of the staphylococcus, it is necessary to take into account the fact that dead cells are destroyed more readily than living ones. The proportion of cells with and without oscillation of leucocytic granules may give a false impression if account is not taken of the fact that the great majority of the cells present in the experimental tubes have disappeared, and especially the dead cells.

In order to avoid any possible toxic effect of the dye and hypotonic salt solution, each sediment is examined immediately after mixture with the solution of dye and the count is effected within 10 min. Tubes are incubated in small batches at intervals so that the period at room temperature after removal from the water-bath between examination of the first and last tubes in each batch is minimal.

The regularity of the results which may be obtained by these methods is indicated in Table I.

RESULTS

Rationale of Method

In our earlier work it was observed that leucocytes suspended in 0·85 per cent NaCl solution deteriorated more rapidly than in Hanks' solution. It was found however, that when a leucocyte suspension obtained with EDTA and dextran was added to Hanks', the tendency to the production of clots which had previously given trouble reappeared.

Zucker and Borrelli (1958), who have made a special study of the effect on blood coagulation of a variety of metallic compounds of EDTA, state that only compounds of EDTA such as those of Na and Mg, in which the basic ion is combined less firmly than Ca are capable of acting as anticoagulants, *e.g.* the compounds of Mn and Ni are not anticoagulants. They point out further that the Na compound of EDTA used in the concentration recommended is peculiar in prolonging the thrombin clotting time of plasma and in causing the platelets to assume a spherical shape and to be readily resuspended after centrifugal sedimentation. It was obvious that the tendency to clotting observed on adding suspensions of leucocytes derived from EDTA treated blood to Hanks' solution was likely to depend on the Ca salt present in the latter.

Observations were therefore made on the effect of adding EDTA in varying amount to Hanks' solution and incubating tubes containing 1 ml. of each of those mixtures to which 2 drops of a suspension of leucocytes in 20 per cent plasma in 0·85 per cent NaCl had been added. The results of these observations are given in Table I.

It is clear from the results recorded in Table I that EDTA is not actively deleterious to the leucocytes and that 1–1000 adequately checks the clotting tendency and so permits of rapid and more accurate counts of the proportion of cells showing active oscillation of granules and resistance to colouring by methylene blue.

If the toxic substance under investigation is likely to have its action controlled by a chelating agent, a duplicate observation on leucocytes suspended in 0·85 per cent NaCl solution will be advisable, although the period in which the leucocytes remain intact in 0·85 per cent NaCl is rather less.

TABLE I.—*Action of EDTA on Leucocyte Suspension in Hanks' Solution*

Conc. of EDTA in Hanks' sol.	Clotting	O.L.G.*	Colour and abundance of granular cells lying free in film
1–250	*Nil*	19/25	Cells ++ and colourless except a few showing trace of blue in nucleus.
1–500	,,	21/25	As above.
1–1,000	,,	16/25	,, ,,
1–2,000	Some small clumps	21/25	Cells + and active cells colourless.
1–5,000	Partial	18/25	As above.
Control I	+†	19/25	,, ,,
Control II	+†	19/25	Cells scanty : about 50 per cent of active cells with light blue nuclei.

* Indicates Oscillation of Leucocytic Granules (the proportion of granular leucocytes in which oscillation of the granules was observed).

† Coagulum visible to the naked eye had been formed.

TABLE II.—*Stability of Leucocytes in Different Suspending Fluids*

Suspending fluid	Period of incubation (hr.)	O.L.G.*	Abundance and colouring of cells
0.85 per cent NaCl	2	20/25	Active cells ++ and uncoloured.
	5	15/25	,, ,, ,, ,, ,,
	20	11/25	Active cells + sometimes coloured : not amoeboid in form.
Hanks' solution	2	8/12	Cells very scanty and slightly stained.
	5	18/25	Cells scanty and slightly stained.
	20	20/25	Most cells in clots : nuclei not well defined.
Hanks' sol. + 0·1 per cent EDTA—neutralized	2	17/25	Cells + : nuclei light blue.
	5	17/25	Cells + : colourless.
	20	14/25	Cells + : O.L.G. very rapid : nuclei defined but not stained.

* See Table I.

In Table II the persistence of activity and character of films obtained after considerable periods of incubation in 0·85 per cent NaCl, in Hanks' solution and in Hanks' with EDTA are shown.

From this it is obvious that the leucocytes are little changed after 5 hr. incubation in any of these fluids but that after 20 hr. the leucocytes appear to be most healthy in the neutralized solution of Hanks' with 0·1 per cent EDTA.

Toxicity of Dye to Leucocytes of the Granular Series

As mentioned above the leucocytes in the control tubes occasionally showed evidence of deterioration if left for more than 1 hr. in contact with dilute solutions of neutral red before examination, Sabin (1923), in a study of vital staining of leucocytes, had found that although she got excellent results with a special preparation of neutral red then supplied by Grübler, the preparations which were available from other sources were more toxic to the leucocytes.

In our earlier work interesting papers of Hope-Simpson (1942 and 1943) on the oscillation of leucocytic granules and the capacity of leucocytes in which

oscillation occurred to reduce methylene blue had been overlooked. In view of these results and of the possible toxicity of neutral red, we have made observations at room temperature on the effects of exposure of leucocytes to solutions of these 2 dyes in hypotonic salt solution.

The results of these investigations are recorded in Table III.

It is clear from these observations that methylene blue is less toxic to the leucocytes than neutral red. It has been adopted for this reason and because its use brings 2 vital phenomena under observation simultaneously—the oscillation of granules and the reduction of methylene blue. The capacity of staphylococcal toxin to prevent reduction of methylene blue by leucocytes, first observed by Neisser and Wechsberg (1901), is widely recognized as a test for leucocidin.

TABLE III.—*Effect of Dyes Dissolved in Hypotonic NaCl Solution on Leucocytes at Room Temperature*

Dye	Concentration	Period of exposure	O.L.G.*	Remarks
Neutral Red	1–2000 in 0·4 per cent NaCl	10 min.	15/25	Active cells mostly colourless, a few with light orange nucleus. Still cells—deep orange nuclei.
	Ditto	50 min.	0/25	All nuclei deep orange.
	,,	2 hr.	2/25	Cells with O.L.G. are only slightly stained and are compact with oscillation of granules which is just discernible.
	,,	20 hr.	<1/25	
Methylene Blue	1–2000 in 0·4 per cent NaCl	10 min.	14/25	Active cells nearly colourless. Still cells frankly blue in nucleus and granules.
	Ditto	50 min.	10/25	Active cells have light blue colouring of nuclei.
	,,	2 hr.	9/25	Active cells all with frankly blue nucleus and granules; in several only a small percentage of granules are oscillating.
	,,	20 hr.	9/25	Active cells with blue or light blue nuclei and granules. Many show considerable protoplasmic protusions (? pseudopodia) in which O.L.G. is marked.

The leucocyte suspension was added 3 drops to 1 ml. of solution of dye and spun down 10 min. after mixing. A small drop of the sediment was transferred to a slide, covered and the coverslip luted to the slide with vaseline. Thereafter repeated observations were made of the cells at the time intervals recorded.

All observations were carried out at room temperature (about 20°).

* See Table I.

DISCUSSION

In considering these phenomena Hope-Simpson (1943) draws attention to the very close connection between active oscillation of granules and capacity to reduce methylene blue and describes how the cell granules may be seen to pass very rapidly from active movement to complete immobility with a simultaneous development of colour. This we have observed occasionally and the close con-

TABLE IV.—*The Comparison of the Leucocidal Effect of Different Salts of Mercury*

Preparation and concentration	O.L.G.*	Colouring of cells, etc.
$HgCl_2$		
1–100,000	0/25	All cell nuclei stained blue.
1–500,000	18/25	Cells with active granules colourless. Cells with stationary granules have blue nuclei.
Thiomersalate		
1–100,000	0/25	Cells mostly with coloured nuclei: a few with uncoloured nuclei.
1–250,000	0/25	Most of cells uncoloured.
1–500,000	2/25	Most of cells uncoloured.
Penotrane		
1–100,000	0/25	Cells mostly heavily stained: a few uncoloured.
1–500,000	2/25	Cells with O.L.G. colourless: all others with blue nuclei.
Mercurochrome		
1–4,000	0/25	Cells mostly stained red but some nuclei light blue.
1–8,000	3/25	Cells with stationary granules have blue nuclei.
Control	19/25	Cells with O.L.G. are uncoloured.

* See Table I.

nection between reduction capacity and granular oscillation strengthens the conclusion that the oscillation is a vital phenomenon since its failure coincides with the failure of an essential respiratory ferment, dehydrogenase.

Hope-Simpson's observations were made, however, with stronger and presumably toxic concentrations of methylene blue, and he was dealing with exudates which must have included a considerable proportion of damaged cells.

In our own observations there have been occasional exceptions to the correspondence between the 2 phenomena. This is indicated by the presence in many of the results recorded of cells showing oscillation of leucocytic granules with some colouration, usually light—Tables I, II and III—and the reverse, *i.e.* absence of staining in granular cells which show no oscillation of leucocytic granules. The latter is exemplified in the leucocytes treated with 1–500,000 thiomersalate in Table IV, in which the toxicity to leucocytes of a number of salts of Hg are compared.

It seems most likely that some substances like thiomersalate may, in certain conditions, prevent penetration of the leucocyte by the dye and that the persistence of movement in cells partly coloured (Table III) indicates persistence of one of the vital activities in a moribund cell.

Sabin (1923) maintained that even the lightest colouring of the nucleus indicated an early stage in death of the cell and without doubt the granular leucocytes when recently separated from the blood and examined by the technique described above show a high proportion of cells which have actively oscillating granules and well-defined nucleus which is quite devoid of colour: but it is obvious from the results recorded in Table II that cells which have earlier shown very light colouring of the nucleus may later fail to show such colouration while very active oscillation of their granules continues.

It is suggested by Sabin (1923) and by Gladstone and Van Heyningen (1957) that the oscillation of leucocytic granules is an agonal rather than a vital phenom-

enon, and that the true vital movement of the granules is a streaming one. This view accords ill with Hope-Simpson's (1943) observation of the close correlation between the oscillation of granules and ability to reduce methylene blue, and with our own that the movement can be observed in leucocytes stretched out in amoeboid form and adapted to foreign cells or bodies. Gladstone and Van Heyningen describe a globular cell containing actively oscillating granules and occasionally snaring off protrusions of protoplasm which persist for a time as small globules full of oscillating granules but without nucleus. Such cells have often been observed and are very probably moribund.

SUMMARY

The yield of leucocytes from fresh normal human blood obtained by the methods described in this paper has been invariably greater than that obtained by the method of Minor and Burnett (1948) which had been used on more than 100 occasions in the course of the last 3 yr. and had, up till now, proved superior to all others tried. It has the further advantage that the leucocytic sediment is easily dispersed so as to give a smooth suspension with a large proportion of separate cells. In addition all the reagents are already available or can be prepared in a sterile condition and bacterium-free suspensions of leucocytes are easily produced.

There is ample evidence that the granular leucocyte which remains colourless in weak solutions of methylene blue in hypotonic salt solution and shows active oscillation of its granules is a living cell. Cells which stain frankly in these conditions but still show some oscillation of their granules are, in all probability, moribund.

Cells which show no movement of granules in hypotonic salt solution are dead and in most instances such cells are heavily stained.

The method of applying these observations in tests of leucocidal activity are discussed. Where the lethal agent is leucolytic as well as leucocidal the total reduction of cells must be taken into account.

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THE ABSENCE OF LEUCOTOXIC ACTIVITY IN HIGHLY PURIFIED DIPHTHERIA AND TETANUS TOXINS (AS BEARING ON THE VIRULENCE OF THE BACTERIA CONCERNED)

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THE methods used in determining the leucocidal effect of a variety of antiseptics, antibiotics and other drugs have been described in an earlier paper, McLeod and McLeod (1959), and improvements of these methods and their application to the study of staphylococcal leucocidin have been discussed, McLeod and McLeod (1961). As these ways of observing leucocidal effects appeared to offer a more exact and simple method than those used previously, a further investigation of some of the bacterial toxins available was made. A number of observations were made on the supernatants separated by centrifugalizing of suspensions in 0·85 per cent NaCl solution of the bacterial growth obtained on cellophane membranes covering solid media—the technique of Birch Hirschfeld (1933)—in this way it was found possible to detect leucocidal activity in many cultures of staphylococcus and in those of the few strains of *Pseudomonas aeruginosa* examined : but it was not successful in demonstrating leucocidin in such cultures of *Corynebacterium diphtheriae* as were tried.

Leucotoxic effects of filtrates of serum broth cultures of streptococci, which had already been observed by staining in the usual way (Channon and McLeod, 1928), were also observed using the methods referred to above. In view, however, of the high purification of diphtheria and tetanus toxins which has been achieved in recent years there was special interest in investigating these, and through the kindness of Dr. C. G. Pope and Dr. R. Thomson of the Wellcome Laboratories at Beckenham, preparations of highly purified tetanus and diphtheria toxins have been placed at our disposal.

It is the purpose of this paper to present the evidence on which the conclusion is based that purified tetanus and diphtheria toxins are devoid of leucocidin. As far as the toxin of the *Clostridium tetani* is concerned the finding falls so much into line with all our knowledge of this disease that the literature need not be discussed.

In the case of diphtheria it is different. The problem of the equipment of the *C. diphtheriae* for the invasion of the human or animal body has been the subject of innumerable experimental investigations and published papers for nearly three-quarters of a century, and is still the subject of controversy.

The main cleavage of opinion is between those, probably the majority, who consider that every feature of the disease can be related to a single toxic substance and the response to that substance in man and animals by antitoxin production. This substance is considered to be neurotoxic, dermonecrotic and lethal. On the other hand there are many observers who cannot satisfy themselves that all

features of the disease can be explained in this way and a smaller group who maintain that they have demonstrated toxic elements derived from the *C. diphtheriae* which are distinct from the classical toxin and important in the development of many of the more formidable cases of the disease.

Zironi (1941) accepts, as everyone must, that there is a great body of evidence for a close correlation between increase in serum antitoxin, the Schick negative state and resistance to the clinical disease. He points out, however, that observations have been repeatedly recorded of (*a*) the development of diphtheria in individuals whose sera were shown to contain considerable amounts of antitoxin in the earliest stages of the disease, and also (*b*) the occurrence of positive Schick reactions in individuals whose sera were well-provided with antitoxin. He concludes soundly that an explanation of any phenomena of disease must satisfy both the usual and the exceptional developments in connection with that disease. He goes on to argue for the occasional development of cellular hypersusceptibility to diphtheria toxin as the reconciling factor. This, he supposes to render the cells reactive to toxin-antitoxin complexes, which would produce no effect on animals with tissues of normal or average susceptibility. One of the epidemiological aspects of diphtheria which has been found difficult to reconcile with the view that classical toxin and its antitoxin are the only factors at play in the manifestations of this disease is the occurrence in certain outbreaks of numerous cases in those immunized with toxoid. Notable examples of this are the Gateshead epidemic on Tyneside, 1939, recorded by Hartley *et al.*, (1950), and the Dundee epidemic, 1941–44, recorded by Tulloch and his colleagues (Hartley *et al.*, 1950) with 45 per cent of all cases in the inoculated. Again Edward and Allison (1951), investigating a smaller outbreak in S. Wales, observed a high incidence in the inoculated.

A further surprising feature of the outbreaks just described has been the existence of moderate or even high titres of antitoxin in the patients' blood serum in the early days of the disease. Hartley sought the explanation of these paradoxical findings in a peculiarly rapid rise of the circulating antitoxin where the first traces of toxin absorbed as the *C. diphtheriae* entered the tissues acted as a booster inoculation on the tissues of an individual already immunized. Tulloch, dealing with similar observations, found it difficult to accept such an explanation since it could not cover the well-established observation of the existence of long-term carriers of virulent *C. diphtheriae* who never have any considerable concentration of serum antitoxin. Hence he is of the opinion that the possibility of a second toxic factor cannot be excluded.

It is the observations of Edward and Allison (1951), however, which are most difficult to reconcile with the supposition that all features of clinical diphtheria depend on an unique toxin. They observed 17 cases in persons immunized or having had a previous attack of disease (1 severe, 6 moderate, 9 mild and 1 very mild) from which they isolated strains of *C. diphtheriae* which even on testing in 4 separate laboratories were consistently found non-toxigenic. The patients had high serum antitoxin levels at the outset of the disease which did not increase in its course. The cases observed by Hartley *et al.*, (1950), and by Tulloch *et al.*, were preponderantly gravis infections, but Edward and Allison's cases (1951) were infected with gravis, mitis and intermedius variants of the *C. diphtheriae*.

Much elaborate work has been done in recent years on the purification of diphtheria toxin by Pappenheimer (1955, 1957) in the U.S.A., by Pope and

Stevens (1958*a*, *b*, *c*, *d* and 1959) in this country and by Relyveld in France, each wording with one or more collaborators. Without attempting to review this literature in detail it may be said that it affords ample evidence of the presence of multiple antigens in crude preparations of toxin and leaves grounds for supposing even crystalline toxin preparations to be complex antigens.

Among recorded observations on the production of toxic substances other than the classical and specific toxin by the *C. diphtheriae* those of O'Meara (1940) have probably attracted most attention. His observations were made on 2 strains of *C. diphtheriae*, the PW8 strain so widely used in toxin production and a "gravis" strain isolated from one of a group of hypertoxic cases of diphtheria which were occurring in Dublin at that time. The crucial finding in this work was that the subcutaneous introduction in the guinea-pig of 0·7–0·5 of a MLD of PW8 toxin and 0·1–2·0 ml. of a saline extract of a 48-hr. Löffler slope of the gravis strain freed of bacteria by centrifugalization and filtration produced a huge local lesion with oedema of wide extent and subsequent necrosis. Neither the PW8 toxin nor the saline extract alone could produce a lesion of this kind (*i.e.* simulating that of hypertoxic diphtheria—" bull-neck ") even in considerably larger doses. Nor could such lesions be produced by PW8 bacilli + toxin. McClean (1941), working with a wide range of strains which included 2 of the Dublin ones, and Zinnemann (1943) who also included Dublin strains, were, however, unable to confirm O'Meara's results.

In the more recent literature, this subject is further explored by Amies (1954) who supposes that O'Meara's results depended on the injection of small numbers of toxigenic *C. diphtheriae* together with a sub-lethal dose of toxin. He was able to kill guinea-pigs by injecting 50,000 toxigenic bacilli (mitis strain) with 0·7 MLD of PW8 toxin, whereas 10^8 of the toxigenic strain alone were required to produce the same result. This criticism depends for its validity on the assumption that O'Meara's " bacteria-free " saline extracts contained small numbers of bacilli. It is unfortunate that Amies had no gravis strains at his disposal and that O'Meara's results were not controlled by parallel experiments with mitis strains recently isolated from clinical cases.

Niggemeyer (1955*a* and *b*) has extended this field of investigation, insisting on the use of media which on account of their iron-content are unsuitable for classical toxin production and also on the importance of using recently isolated strains. He compared a diphtheroid strain from croup, a mitis strain from laryngeal diphtheria, an intermedius strain from 1 of 2 associated cases, of which the other was fatal, and a gravis strain from a case of peculiar severity. With all 4 he got evidence of production of a necrotizing factor, but in the case of the gravis strain the killing-power of broth cultures for guinea-pigs was outstanding and when they were protected from death by 1,000 units of antitoxin a local reaction and necrosis still occurred. In media containing excess of iron the ratio of *in vivo* activity of the toxins produced was of 1,000 to 1 in favour of the gravis as compared with the PW8 strain. He claims that O'Meara's B factor is a complex of the necrotizing element, which he has isolated, and hyaluronidase. The spreading factor is best demonstrated in media containing hyaluronic acid and antitoxin to neutralize other toxic factors. Further support for O'Meara's contention appears in recent work by Oehring (1959) who suggests differences in production of necrotizing toxin and specific lethal toxin in explanation of discrepancies between the results of gel diffusion and Römer tests for virulence when they are applied to large

collections of strains. And Eisler (1958) observes greater loss of lethal than of necrotizing toxin when a culture of *C. diphtheriae* in a " synthetic " medium is filtered with a Seitz disc.

Again Poulik and Poulik (1958) claim to separate 2 major factors from diphtheria toxin by electrophoresis in a starch gel, one killing at 1–1,000 dilution, the second only killing undiluted but causing greater skin necrosis than the first. Finally Branham, Hiatt, Cooper and Riggs (1959), employing the methods of immune electrophoresis and ultra-centrifugalization, claim to isolate from a " gravis " strain a component of high molecular weight which was not recognized in PW8 toxin and produced marked phenomena of oedema in experimental animals. Cumulatively, therefore, there is a formidable amount of evidence for the existence of toxic factors, produced at any rate by some strains of *C. diphtheriae* which are distinct from the classical specific toxin and of significance in the disease.

Ørskov, Andersen and Poulsen (1944), impressed by the severity of the " gravis " diphtheria which had appeared in Denmark towards the end of the 1939–45 war and by the peculiar pathogenicity for the guinea-pig of the gravis strains which were then isolated, made an elaborate study of the local reaction. They observed that the lesions due to " gravis " strains contained few leucocytes and these were not actively phagocytic, whereas by contrast lesions due to " intermedius " strains were full of leucocytes which showed considerable phagocytic activity. Further, 10 times as much toxin could be squeezed out of the tissues of local gravis lesions as from intermedius lesions. Finally the simultaneous administration of 10^7 bacilli and 100 units of antitoxin was followed by relatively active phagocytosis. The largest doses of antitoxin did not, however, eliminate vascular reaction, oedema and cellular degeneration at sites of injection.

This careful work of Ørskov *et al.*, obviously suggests that the invasive power of a diphtheria strain depends on its ability to produce the classical toxin *in vivo* and that the leucocytes are highly susceptible to such toxin, although it does not necessarily exclude the possibility that a second toxic factor is produced much more readily by the more formidable strains *in vivo*.

In view of these conflicting opinions, the opportunity afforded recently of examining much more highly-purified samples of toxin and having at our disposal a new and highly sensitive method of testing the leucocidal qualities of toxin, we sought to explore this particular aspect of the problem afresh.

Observations on the Leucocidal Action of Highly-Purified Tetanus and Diphtheria Toxins

The methods of these observations have been described by McLeod and McLeod (1959 and 1961), but were for the most part done with the first and more laborious method.

If the number of cells showing active OLG in a given tube were less than 50 per cent of those in the control it was assumed that some deleterious effect was being shown and the complete absence of " active " cells was accepted as definite proof of leucodidal activity in the substance investigated and at the concentration used.

The first observations made on these lines are recorded in Table I.

The diphtheria toxin was a crystalline product of high purity—over 3,000 Lf/mg.N. The tetanus toxin was a solution containing 775 Lf/mg.N and had been made up from a preparation of a potency of 2680 Lf/mg.N. The indication of

TABLE I.—*Leucotoxic Effect of (Pure) Diphtheria and Tetanus Toxins*

Concentration w/v of *diphtheria* toxin in Hanks' solution to which leucocytes were exposed.	Proportion of leucocytes showing active granule oscillation when exposed subsequently to hypotonic NaCl solution
1—100	0
1—500	0
1–1,000	2/25
1–2,000	19/25
Control	20/25
Concentration of tetanus toxin in Hanks' solution	
1–3,000	0
1–6,000	3/25
1–12,000	10/25
Control	9/25

these observations was that the toxins were deleterious to leucocytes but only in concentrations which contained many MLD for small experimental animals.

It seemed therefore desirable to demonstrate that the effect was, in fact, due to diphtheria toxin and could be neutralized by specific antitoxin. Observations on this point are recorded in Table II.

TABLE II.—*Equivalent Neutralization of Diphtheria Toxin by Tetanus and Diphtheria Antitoxins*

Concentration of diphtheria toxin* 1–500 w/v	Added antitoxin (undiluted) Tetanus	Added antitoxin (undiluted) Diphtheria	Diluent Hanks' soln.	Result O.L.G.	Result Other phenomena
0·5	*Nil*	*Nil*	0·5	0/25	Cells much ballooned.
0·5	0·25	—	0·25	15/25	
0·5	0·04	—	0·46	2/25	Considerable ballooning.
0·5	—	0·25	0·25	14/25	
0·5	—	0·05	0·45	0/25	Cells much ballooned.

* This toxin had contained 1–10,000 merthiolate originally but had presumably lost some part of this in dialysis prior to freeze-drying.

O.L.G. = Oscillation of leucocytic granules.

It is clear from the observations in Table II that the capacity of antiserum to reverse the toxic effect on leucocytes is very limited and in no way specific, hence it is more likely to be a protein effect than the effect of antitoxin. A further line of investigation with these toxins which was likely to throw light on the nature of the deleterious effect on leucocytes was to determine the thermolability of the toxic substance.

For this purpose an investigation was made with the tetanus toxin as recorded in Table III.

It is clear that the substance toxic to leucocytes is not thermolabile and hence not specific toxin. The effect of heating the toxin of *C. diphtheriae* is reported in the footnote to Table V.

In the course of the observations on antitoxin neutralization commercial antitoxin had at first been used but on account of the fact that it was found to be toxic to leucocytes at considerable dilution and that thiomersalate, the active component in the preservative which it contained, had been shown to be toxic to

TABLE III.—*Absence of Thermolability of Toxic Factors for Leucocytes in Preparations of Tetanus Toxin*

Concentration of toxin w/v	Preservative	Impurity	Result: O.L.G.	Result: Other phenomena
1–3,000	None	Trace of Cd.	<1/25	Considerable ballooning.
1–6,000	,,	,, ,,	7/25	Some ballooning.
1–12,000	,,	,, ,,	13/25	—
After heating of toxin for 30 min. at 65°				
1–3,000	None	Trace of Cd.	0/25	—
1–6,000	,,	,, ,,	6/25	Ballooning marked.
1–12,000	,,	,, ,,	15/25	Some ballooning.

leucocytes even in dilutions as high as 1–100,000 or more, it had been found necessary to obtain antitoxin free of preservative to carry out the observations recorded in Table II.

It also appeared from these observations that the presence of preservative or other impurities in the toxin preparations might explain their effect on the leucocytes.

On discussing this possibility with Dr. Pope it was ascertained that the crystalline diphtheria toxin was likely to contain traces of thiomersalate which it would be difficult to measure exactly and that the tetanus toxin contained traces of cadmium salt.

We are indebted to Dr. Pope for supplying a sample of diphtheria toxin and of antitoxin free from preservative with which to investigate these points.

The results of the observations made are recorded in Tables IV and V.

TABLE IV.—*Comparison of Leucotoxic Effects of Diphtheria Toxins With and Without Preservative*

Toxin	Dilution in Hanks' soln.	Preservative	O.L.G. after different time exposures at 37°: 1 hr.	3 hr.	4 hr.
MS.710	1–500	thiomersalate	0/25	Not examined	
MS.1170	1–500	None	20/25	13/25	13/25
Nil	Control	—	17/25	12/25	5/25

TABLE V.—*Leucotoxic Effect of Diphtheria Toxins With and Without Preservative Working at Different Concentrations*

Toxin	Concentration w/v	Preservative	Result: O.L.G.	Result: Other phenomena
MS.710	1–200	thiomersalate	0/25	Mostly debris.
	1–1000	,,	14/25	Cells showing movement are swollen.
	1–5000	,,	15/25	—
MS.1170	1–200	None	22/25	—
	1–1000	,,	22/25	—
	1–5000	,,	18/25	—
Control	—	—	20/25	—

Duplicate series in which toxins were heated at 65° gave results similar to those recorded above.

It is reasonably proved by these observations that the preservative present in the toxins is responsible for the leucotoxic effects which they produce and hence that specific and highly-purified diphtheria and tetanus toxins are devoid of leucotoxic effect for cells of the granular series.

DISCUSSION

It is considered that the evidence already produced by Hope-Simpson (1942 and 1943) and by ourselves (1959 and 1961), proves that the tests used are good evidence for toxic action on leucocytes : and the presence of oscillation of granules in leucocytes as evidence of life has been accepted by Sabin (1923), Hansen Pruss (1936) and Minor and Burnett (1949), as well as by a number of French workers quoted by the authors in a previous paper (1959). It may, of course, be that a more delicate test will detect an injurious effect missed in these observations, but this seems improbable. A remotely possible source of error was destruction of toxin owing to conditions of transit by the post office. This possibility was excluded by direct test on the guinea-pig, which was readily killed by quite small amounts.

If the specific lethal toxin of the *C. diphtheriae* were toxic to the leucocytes, then it would be easy to accept the suggestion that this toxin was the only important factor in invasion of the body. It appears, however, from the above observations that the leucocytes of the granular series are resistant to it. It may still be argued that the influence of the toxin on other elements in the tissues, the fixed tissue cells or the autonomic nerves controlling the circulation in the finer blood vessels may so modify the local reaction as to facilitate invasion of the body by the bacillus. Certainly it is claimed that diphtheria toxin is lethal to some cellular elements in tissue culture. Placido Sousa and Evans (1957), La Placa (1957), Penso and Vicari (1959).

Modification of the local reaction has seemed all-important to Ørskov and to Amies (1954), and Agarwal and Hole (1959) have drawn attention to the diminished sensitivity of the sympathetic nerve endings in the involuntary muscle of the smaller blood vessels as the cause of the capillary dilatation brought about by diphtheria toxin.

On the other hand is all the evidence for the production of toxic elements other than specific lethal toxin by the diphtheria bacillus, already referred to. The observations recorded in this paper weaken the case for supposing the specific toxin to be the sole significant weapon of the *C. diphtheriae*, although they do not bring any direct evidence for a secondary toxic factor distinct from the classical toxin.

SUMMARY

No evidence has been obtained that purified diphtheria toxin or tetanus toxin exerts a leucocidal effect on cells of the granular series. The evidence for this is that polymorphonuclear leucocytes suspended in hypotonic salt solutions containing 1-2000 methylene-blue remain uncoloured and full of granules in a lively state of oscillation after exposure to high concentrations of toxin containing many MLD for the guinea-pig. Such leucotoxic effects as have been observed with solutions of diphtheria and tetanus toxins are due to the preservative or other impurity which they contain.

It seems improbable that the wide variations in virulence among strains of *C. diphtheriae* can be fully explained by varying ability to produce the classical lethal toxin.

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Thanks are due to Dr. Cumming for help in procuring samples of blood ; also to Mrs. Greenan and Miss Alison Knox for ready and effective technical assistance.

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SURVEY OF PAPERS

SUTTON and TYRRELL prepared interferon in human, simian and bovine tissue cultures using live influenza A virus. They tested their preparations for capacity to protect homologous and heterologous tissue cultures against ECHO 11 or parainfluenza 1 viruses. They found that interferon made in monkey kidney culture protected heterologous tissues; protection by other interferons was not universal or was confined to cells of the species of origin. They also found that protection depended on overnight contact of cells with interferon, and lasted 5–8 days. Activity of the material survived freeze-drying provided that 0·1 per cent gelatin was added to it (p. 99).

COUCH, MCBRIDE, DAMMIN and MURRAY, using dogs performed nephrectomy and after an interval of 3 months transplanted the remaining kidney to the iliac vessels in one group and in the other group performed a simultaneous nephrectomy with autografting of the remaining kidney. At monthly intervals following operation a laparotomy was performed to measure the kidney size and to take a renal biopsy. In addition multiple tests for renal function were performed. These experiments were terminated by killing the animals and immediately examining the kidneys macroscopically and microscopically.

It was found that renal compensatory hypertrophy occurred in the autograft if the contralateral kidney was removed at the time of the autograft but no significant further increase in size occurred in the kidney which had been solitary for 3 months and was subsequently autografted. There was histological evidence of nerve regeneration at 3 months which appeared complete at 6 months. The serial renal biopsies and the eventual kidney examinations revealed no constant histological abnormalities and the renal function tests remained within normal limits in the renal autografts (p. 106).

WYNN and BALL have found no change in distribution or activity of rib cartilage or liver succinic dehydrogenase in experimental lathyrism (p. 114).

MASON and KAUFFMAN studied the toxic effect of NDV on the cultured cell strains L, U_{12}, $LLCM_1$, KB and S180. They found that adsorption of virus to cell necessary for subsequent toxic effect occurred equally well at 4° or 35°, and, after 3 min., could not be reversed by immune serum. RDE prevented adsorption if at least 500 units were applied to each culture of $3–4 \times 10^5$ cells. On heating or U.V. irradiation, viral properties were lost in the following order: infectivity, toxicity, haemagglutinin (p. 118).

WATSON having studied the influence of experimental hypercholesterolaemia on serum lipoprotein distribution in rats concludes that the changes observed are associated with the formation of an unstable and hitherto uncharacterized α-lipoprotein (p. 131).

PRIORESCHI and SELYE examined the hearts, kidneys and stomachs of 60 female Sprague-Dawley rats following gastric administration of calcium acetate and forced restraint in some of them by fastening the animals in the prone position by adhesive tape. All the animals showed necrotozing gastric lesions but only those on a high dosage of calcium acetate with or without forced restraint produced nephrocalcinosis, coronary artery calcification of the Monckeberg type and calcified streaky necrosis of the myocardium. They concluded that stress plays a decisive role in the production of this cardiopathy (p. 135).

OSHIMA, MYRVIK and EVA LEAKE produced granulomata in rabbits' lungs by the intravenous injection of BCG in saline 6 weeks after vaccination at 2 inguinal sites with heat-killed BCG in Freund's adjuvant.

Extracts of such granulomatous lungs proved up to 8 times more inhibitory against BCG than those of normal lungs. Cellulose column fractionation of the extracts showed that the

actively bacteriostatic fraction also contained most of the increased quantity of lysozyme found in granulomatous lungs (p. 138).

KATO and HARA have shown that large intravenous doses of influenza A (PR8) kill rabbits without evidence of virus multiplication in the animals' tissues. This toxic property of the virus, together with its infectivity for eggs, is destroyed by heat and U.V. light, but, unlike infectivity is not affected by prolonged sonic vibration (p. 145).

WILLIAMS, GRIFFEN, SHARMA and WANGENSTEEN have examined the composition and volume of secretions from parts of the rabbit gut where aggregations of lymphoid tissue are found. They found that the secretions of the appendix and the saccus rotundus were identical, serous, alkaline and rich in bicarbonate ions. They deduce that the cells responsible for the secretion are those epithelial cells in close relation to the lymphoid tissue (p. 153).

FESTENSTEIN and BOKKENHEUSER inoculated pregnant rabbits subcutaneously with *Treponema pallidum* (Nichol's strain). The young showed no sign of disease or of immune tolerance towards the organism. Inoculation of rabbits within 5 hr. of birth caused an illness progressing to death within 16 weeks. Arrest of this illness with penicillin 5 weeks after infection did not lead to immune tolerance (p. 158).

GRESHAM and HOWARD fed rats on a diet containing 40 per cent fat, 5 per cent cholesterol, 2 per cent cholic acid and 0·3 per cent thiouracil and the effects of adding natural and synthetic fats were investigated. It was found that thrombosis was produced with butter, beef fat, hydrogenated arachis and synthetic glycerides containing stearic and oleic acids, whereas arachis oil resulted in atherosclerosis without thrombosis.

Mixtures of butter and arachis oil, butter with 8 per cent methyl trioleate and maize oil produced a combination of both thrombosis and atherosclerosis while glyceryl trioleate and trilinoleate produced neither.

They concluded that saturated fatty acids are thrombogenic and that linoleic acid in conjunction with fatty acids is atherogenic (p. 166).

JOYCE MCLEOD and MCLEOD have continued their work on leucocidal substances. They have developed a highly satisfactory method of obtaining living leucocytes from blood by using EDTA as an anticoagulant and dextran to sediment the red blood cells. Evidence of viability based on the movement of granules and uptake of stains are examined and the methods of applying these observations to tests for leucocidal activity are discussed (p. 171).

JOYCE MCLEOD and MCLEOD, using the technique previously described, have demonstrated that highly purified diphtheria and tetanus toxins exert no leucocidal effect. They discuss the significance of this at length in relation to the toxicity of diphtheria toxin (p. 179).

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MITOCHONDRIAL DAMAGE AND HEPATIC STEATOSIS IN ETHIONINE INTOXICATION*

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THE relation between injury to mitochondria and fat accumulation in the liver is not established. It has been suggested that the inability of mitochondria to oxidize fatty acids may lead to steatosis (Artom, 1959); conversely, fat accumulation in the cytoplasm of the liver cells may injure mitochondria. However, the 2 processes might be independent of each other. To investigate this relationship, mitochondrial changes and hepatic steatosis were studied during acute ethionine intoxication. It was possible to dissociate the 2 phenomena by variations of dosage, time interval and sex variations as well as by modifications of the ethionine intoxication with simultaneous administration of cortisone, Janus Green, dinitrophenol and methionine. The oxidation of proline, glutamate, succinate, and of fatty acids *in vitro* and *in vivo* as well as the mitochondrial capacity for oxidative phosphorylation was correlated with the changes in lipid concentration of the liver.

MATERIAL AND METHODS

Sprague-Dawley rats, 150 g. in weight, were studied in groups of 6. Females were used except when stated otherwise. Ethionine was administered either by intraperitoneal injection of a 2 per cent aqueous solution or by feeding a synthetic diet (Popper, de la Huerga and Yesinick, 1953) containing 0·5 per cent ethionine.

Liver homogenates were prepared in 0·25 M sucrose solution, and mitochondria were isolated by differential centrifugation (Devlin and Lehninger, 1958). Proline oxidase activity was measured by the rate of disappearance of proline after incubation (Roche and Ricaud, 1955); proline was estimated by the method of Troll and Lindsley (1955). If the oxidative phosphorylation was measured simultaneously, the hexokinase-glucose system was included in the incubation medium (Ernster and Loew, 1955), the oxygen consumption measured in a Warburg flask, and inorganic phosphate was determined by the method of Lowry and Lopez (1946). Oxidation of glutamate (Weinbach, 1959) and stearate (Masoro and Porter, 1960) was determined manometrically. Succinic dehydrogenase activity was estimated by a tetrazolium method as described by Barka and Dallner (1958). The extraction and esti-

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mation of hepatic fat was carried out in accordance with the description of Koch-Weser, Farber and Popper (1951).

For the measurement of fatty acid oxidation *in vivo*, the animals received 2 μc stearic-1-^{14}C acid (specific activity 8·33 μc/mg.) or 1 μc sodium acetate-1-^{14}C (specific activity 25 μc/mg.) intraperitoneally 19 hr. after the ethionine injection. Rats, fasted for 19 hr., served as controls. Their body weight matched that of the experimental rats. The animals were placed in a desiccator immediately after the isotope injection and supplied with CO_2-free oxygen. The exhaled CO_2 was collected for 20 min. in a series of 3 centrifuge bottles, each containing 150 ml. of a solution consisting of 2 parts 10 per cent $BaCl_2$ and 1 part 0·1 N NaOH and 10 mg. phenolphthalein per litre. The resulting $BaCO_3$ precipitate was centrifuged and washed with water several times. The total amount of precipitated CO_2 was determined titrimetrically and its activity measured on a planchet in a gas flow counter. Corrections were made for background and self absorption.

RESULTS

Variation of single doses of ethionine

Groups of rats received ethionine intraperitoneally in doses of 0, 5, 15, 30, 45, 60 and 75 mg./100 g. body weight. They were fasted and killed 24 hr. later. Following administration of 15 mg. ethionine/100 g. body weight, the liver fat doubled ; it was even higher in rats which had received 30 mg. ethionine. A larger dose had only a slightly greater effect. The oxidation of proline by the isolated mitochondria from the same livers was not significantly decreased in rats which had received up to 45 mg. ethionine/100 g. body weight. Following administration of a higher dose of ethionine, the proline oxidase activity declined. Proline oxidase activity was reduced by 50 per cent after injection of 75 mg. ethionine/100 g. body weight. Determination of the efficiency of the coupled phosphorylation showed a similar decline in P/O ratio. The complete oxidation of 1 μmole of proline requires 11 μatoms of oxygen. The values found in the control animals or in those treated with a small amount of ethionine are very close to the theoretical value, but an increase in the ethionine dose produced a decline to 7·6. This together with the decrease to one-half of the mitochondrial capacity for coupled phosphorylation resulted in a decrease to one-fifth of the total amount of high energy phosphates synthesized during the oxidation of the proline (Table I).

TABLE I.—*Effect of Ethionine Intoxication on Hepatic Fat Concentration and on Mitochondrial Proline Oxidation and Oxidative Phosphorylation. Variation of Single Doses of Ethionine*

Ethionine (mg./100 g. body weight)	Proline* (μmoles)	Oxygen* (μatoms)	Pi* (μmoles)	P : O	Oxygen/Proline (μatom/μmole)	Fat (g./100 g.)
None	81·3 ± 8·1	862	1724	2·30	10·6	5·0 ± 1·0
5	76·0 ± 6·2	760	1702	2·24	10·0	6·6 ± 1·3
15	73·8 ± 5·8	753	1611	2·11	10·2	9·9 ± 2·2
30	76·0 ± 9·0	752	1534	2·04	9·9	12·3 ± 3·4
45	77·6 ± 12·0	776	1436	1·98	10·0	11·3 ± 2·8
60	65·0 ± 10·0	623	1009	1·62	9·6	13·0 ± 1·9
75	45·7 ± 4·1	347	336	0·97	7·6	14·1 ± 1·8

* Results expressed per total mitochondria per liver. Each result represents 12 estimations from 6 animals.

Oxidation of glutamate by liver homogenate was at control level with doses of 15, 45 and 60 mg. ethionine, and only 75 mg. ethionine/100 g. body weight

impaired glutamate oxidation. This could be restored by addition of diphosphopyridine nucleotide DPN (Fig. 1).

The oxidation of stearate by isolated mitochondria remained at the control level with doses of 15, 45 and 60 mg. ethionine, and only addition of 75 mg. or more ethionine/100 g. body weight decreased stearate oxidation (Fig. 2).

Effect of time upon the changes induced by a single ethionine injection

Groups of rats were killed 2, 4, 6, 7, 10, 12, 16, 24 and 48 hr. after injection of 75 mg. ethionine/100 g. body weight. Six hours after injection, the liver fat increased by 40 per cent, reached nearly 3 times the control level within 24 hr.,

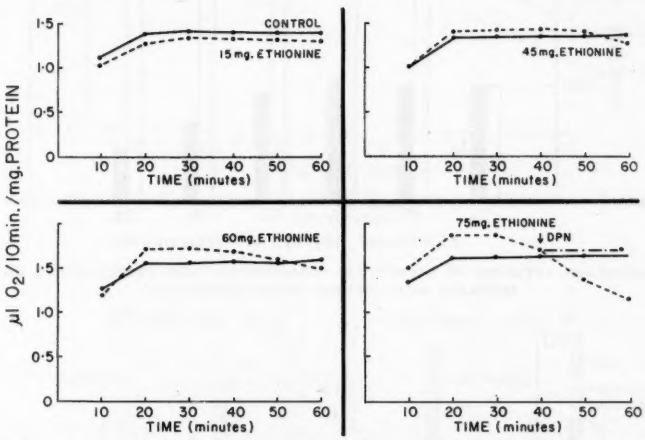

FIG. 1.—Effect of ethionine intoxication on glutamate oxidation in the liver. Variation with single dose of ethionine.

and fell to almost control level by 72 hr. Mitochondrial proline oxidation and oxidative phosphorylation began to decrease by the 12th hr., and continued so for the duration of the experiment (Fig. 3).

Impairment of stearate and glutamate oxidation was apparent 16 hr. after ethionine injection, while succinic dehydrogenase activity began to diminish only after 24 hr. (Fig. 4).

Modifications of the ethionine intoxication

Less hepatic fat accumulated in male than in female rats 24 hr. after injection of 75 mg. ethionine/100 g. body weight. Proline oxidation and oxidative phosphorylation decreased in both males and females at a comparable rate (Fig. 5).

The simultaneous intraperitoneal injection of 15 mg. ethionine and 2·5 mg. cortisone/100 g. body weight resulted in a greater fat accumulation in the liver after 24 hr. than following ethionine injection alone. However, the cortisone administration did not intensify the impairment of the mitochondrial proline oxidation and of the oxidative phosphorylation. On the other hand, if 2 mg. Janus Green/100 g. body weight or 2 mg. dinitrophenol/100 g. body weight was

injected together with 15 mg. ethionine, the proline oxidation and the P/O ratio significantly decreased without further change in hepatic fat content (Fig. 5).

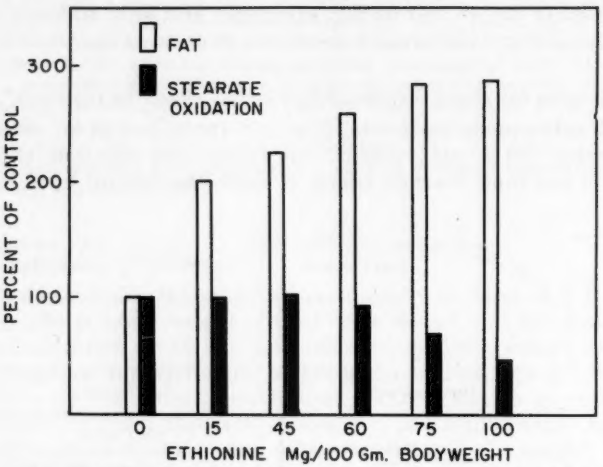

FIG. 2.—Percentage variation of hepatic fat concentration and mitochondrial fatty acid oxidation after different doses of ethionine.

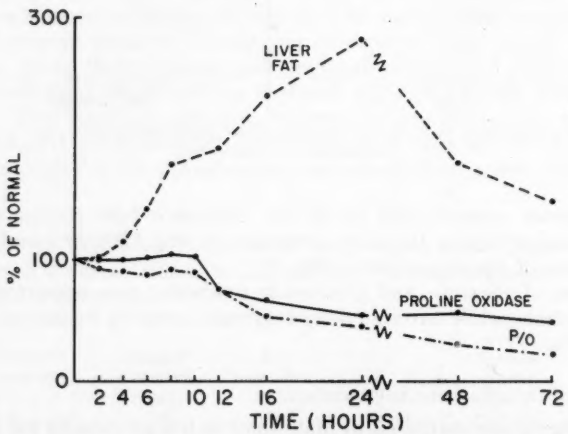

FIG. 3.—Effect of time on hepatic fat concentration, and on mitochondrial proline oxidation and oxidative phosphorylation during ethionine intoxication. Ethionine dose: 75 mg./100 g. body weight.

Effect of feeding of 0·5 per cent ethionine diet

Groups of rats were killed after having been on a diet containing 0·5 per cent ethionine for 3, 7, 14 and 21 days. The liver fat increased 3-fold within 3 days and declined subsequently to control level at 21 days. The mitochondrial oxida-

tion of proline and stearate declined steadily throughout the period of 21 days. Other groups of animals were killed at the same intervals after having been fed an 0·75 per cent ethionine diet supplemented with 0·6 per cent methionine. The hepatic fat concentration was almost equally elevated throughout the entire

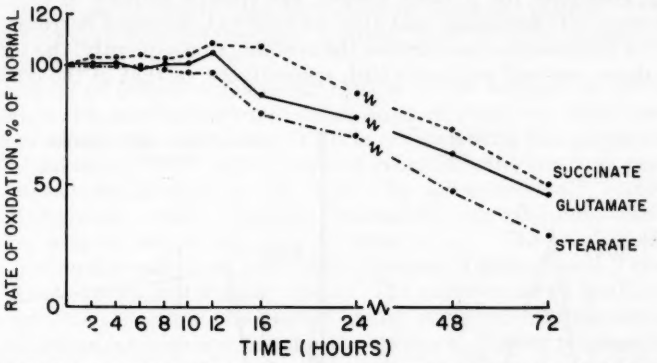

FIG. 4.—Effect of time on the oxidation of succinate, glutamate and stearate during ethionine intoxication.

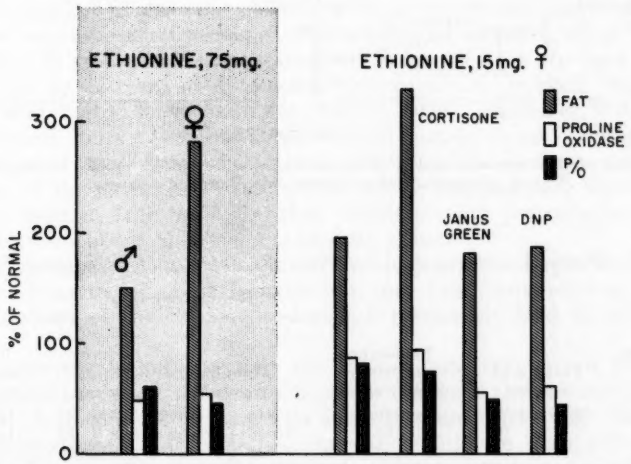

FIG. 5.—Modifications of ethionine intoxication. Changes of hepatic fat concentration and of mitochondrial proline oxidation and oxidative phosphorylation.

experimental period. In contrast the mitochondrial oxidation of proline and stearate remained at control level (Fig. 6).

Oxidation of fatty acids in vivo

To compare fatty acid oxidation *in vivo* with that by the isolated mitochondria *in vitro*, groups of rats were injected with 45 or 100 mg. ethionine/100 g. body

weight 19 hr. before receiving 1-^{14}C labelled stearate or acetate. After administration of 45 mg. ethionine/100 g. body weight, the specific activity of respiratory $^{14}CO_2$ from both labelled stearate and acetate increased. Total activity was at control level with acetate and somewhat higher with stearate. After injection of 100 mg. ethionine/100 g. body weight, the specific activity of CO_2 from both labelled compounds decreased, and also the total CO_2 collected decreased by about one-third for the stearate, one-half for the acetate. Consequently, the total activity was only about one-half and one-third, respectively, of that of the control (Table II).

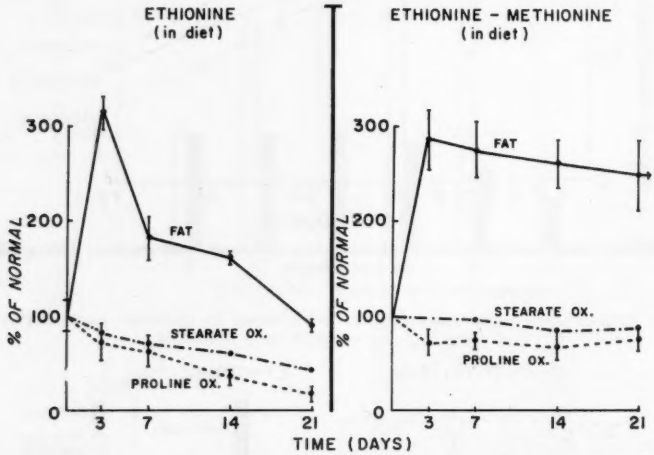

FIG. 6.—Effect of ethionine diet and of ethionine-methionine diet on hepatic fat concentration and on mitochondrial oxidation of proline and stearate.

TABLE II.—*Fatty Acid Oxidation* In Vivo *of Control and Ethionine Treated Rats*

Number of rats	Fatty acid injected	Ethionine (mg./100 g. body weight)	Respiratory CO_2* Amount (μmoles)	Specific activity (c.p.m. per mg.)	Total activity (c.p.m.)
5	Stearic-1-^{14}C	none	0·844 ± 0·200†	305 ± 40	11,326
5	,,	45	0·866 ± 0·190	388 ± 50	14,784
4	,,	100	0·522 ± 0·084	236 ± 31	5,420
5	Acetate-1-^{14}C	none	0·972 ± 0·220	828 ± 105	35,412
5	,,	45	0·848 ± 0·170	1,004 ± 145	37,445
4	,,	100	0·468 ± 0·070	658 ± 90	13,549

* Respiratory CO_2 collected for 2 times 10 min.
† Mean ± standard deviation.

DISCUSSION

Mitochondrial injury and hepatic fat accumulation occur frequently together in toxic hepatic injury. To examine their relationship, accumulation of fat was

correlated with alterations of the activity of enzymes considered characteristic of mitochondria. The ethionine intoxication served as a model since modifications of this intoxication were found to produce independent variations in either hepatic steatosis or mitochondrial injury as reflected in changes of the oxidation of proline, glutamate, succinate, stearate and of oxidative phosphorylation. The results obtained with these different enzyme systems were in close agreement in the various conditions.

The examples of excessive fat accumulation in the absence of mitochondrial damage include the administration of small doses of ethionine, since the amount required to produce steatosis within 24 hr. is one-fourth of the minimum dose to damage mitochondria. With higher doses of ethionine, the steatosis precedes the decline of mitochondrial activity by 6 hr. In agreement with earlier studies (Farber and Segaloff, 1955), cortisone intensified the ethionine effect on fat accumulation without enhancing mitochondrial injury. The same holds true of the known preventive effect of methionine (Jensen, Chaikoff and Tarver, 1951 and Stein, Kent, Barka and Popper, 1960). The supplement of methionine given in this experiment prevented the decline of the mitochondrial functions characteristic of ethionine intoxication but not the steatosis. Under the electron microscope in early stages of steatosis induced by ethionine, the mitochondria appeared not distinctly altered (Grisham, 1960).

In contrast, mitochondrial damage is found in male rats with little steatosis in response to doses of ethionine which produce considerable steatosis in female rats. This sex difference previously demonstrated (Farber, Koch-Weser and Popper, 1951) in steatosis is less pronounced in loss of liver glycogen (Lupu and Farber, 1954), in decrease of microsomal detoxification (Herken, Maibauer and Neubert, 1958), and in hepatic fibrosis (Becker, 1956). Moreover, if Janus Green or dinitrophenol, known to cause mitochondrial damage *in vivo* (Vorbrodt, 1957), is combined with small doses of ethionine, mitochondrial damage ensues without aggravation of the steatosis. These results are in keeping with the observation (Recknagel, Stadler, Litteria, 1958) that adrenalectomy prevents fat accumulation but not mitochondrial damage caused by ethionine.

It appears, therefore, that mitochondrial injury is not required for hepatic fat steatosis, at least in ethionine intoxication, and that accumulation of the fat in the cytoplasm of the liver cells does not necessarily lead to mitochondrial damage.

These experiments indicate that the fat accumulation is not the result of faulty fatty acid oxidation in the mitochondria. Claims to the contrary in ethionine intoxication (Artom, 1959) are based on doses 15 times higher than the minimum dose which produces steatosis and 4 times the minimum dose which produces mitochondrial injury. The rate of fatty acid oxidation *in vitro* is normal during fat accumulation in ethionine intoxication, as also in fatty livers produced by CCl_4 (Artom, 1958). Moreover, the specific activity of respiratory $^{14}CO_2$ from oxidation of radioactive stearate or acetate *in vivo* is increased in rats treated with a relatively low dose of ethionine. These observations suggest a relatively increased energy production by fatty acid oxidation in the cells during steatosis. For this mitochondria are required which might explain the significance of the apposition of mitochondria to fat droplets, demonstrated by electron microscopy (Palade, 1959), which possibly accumulate from fat transport to the site of oxidation (Wertheimer and Shafrir, 1960) to meet energy requirements.

SUMMARY

Mitochondrial changes as reflected in the oxidation of proline, glutamate, succinate and stearate and in oxidative phosphorylation were correlated with the accumulation of fat in the liver during acute ethionine intoxication. Both conspicuous fat accumulation and severe mitochondrial damage were produced by ethionine in doses above 60 mg./100 g. body weight.

Fat accumulated without mitochondrial damage with low doses of ethionine, in the early phase of the intoxication and on an ethionine-methionine diet. The steatosis could be intensified without damage to the mitochondria by simultaneous administration of cortisone.

Mitochondrial damage occurred with only moderate steatosis in male rats treated with high doses of ethionine, or if low ethionine doses were combined with Janus Green or dinitrophenol.

Fatty acid oxidation *in vitro* and *in vivo* were similarly influenced by various experimental conditions.

The results suggest that mitochondrial damage and fat accumulation are separate and independent manifestations of the ethionine intoxication.

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BLOOD COAGULATION STUDIES IN RATS GIVEN DIETS WHICH PRODUCE THROMBOSIS OR ATHEROSCLEROSIS

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VASCULAR thrombosis and myocardial infarction can be produced experimentally by feeding a diet containing 40 per cent butter together with thiouracil, cholesterol and sodium cholate (Thomas and Hartcroft, 1959). If 40 per cent arachis oil is substituted for butter, no thrombosis occurs but extensive atherosclerosis of the aorta and coronary arteries is produced (Gresham and Howard, 1960). These experiments suggest that thrombus formation is not dependent on the development of atherosclerosis but the result of altered coagulability of the blood. The present study reports differences in the standard coagulation tests between rats on normal diets and those fed diets which produce thrombosis or atherosclerosis.

MATERIALS AND METHODS

Animals

Thirty-six laboratory bred male rats, matched according to weight were divided into 3 groups and given diets similar to those described previously (Hill, Howard and Gresham, 1960).

Control group I was given a stock diet containing 10 per cent arachis oil.

Thrombogenic group II was given a diet which produces thrombosis containing 40 per cent butter, 2 per cent cholic acid, 5 per cent cholesterol and 0·3 per cent thiouracil.

Atherogenic group III was given a diet which produces atherosclerosis by substituting 40 per cent arachis oil for butter in the group II diet.

Collection of blood

During the 12 hr. prior to testing, the intake of the rats was restricted to water. Two animals were killed from each group daily after periods varying from 14–90 days on the diets.

Carbon dioxide snow was placed in a plastic container and covered with a fenestrated partition. The animal was placed in the container which was closed with a glass lid. After approximately 45 sec. respirations ceased. Blood was immediately removed from the inferior vena cava after an abdomino-thoracic dissection (Flute and Howard, 1959). In all cases the heart was beating at the time of venepuncture.

Blood was collected in paraffined syringes, mixed with a 20 per cent trisodium citrate solution (0·2 ml./10 ml. blood) and kept at 4° until tested. Studies were restricted by the small volume of blood available, varying from 2·5 ml. in the smaller experimental animals to 7·5 ml. in the larger control rats. The plasma of the experimental animals was invariably milky.

Coagulation tests

The methods in general use are those recommended by Biggs and Macfarlane (1957) and included: Whole blood coagulation times (Lee and White, 1913) in glass and silicone tubes

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(silicone clotting time). A single tube was inverted every 15 sec. Calcium clotting time: Stypven time (Rapaport, Aas and Owren, 1954): One-stage prothrombin time: Two stage plasma and serum prothrombin times (Merskey, 1950): Thromboplastin generation test (Biggs and Douglas, 1953): Thromboplastin screening test (Hicks and Pitney, 1957).

Direct platelet counts were carried out using a 1 per cent sequestrene solution with cresyl blue. Direct platelet counts in hyperlipaemia present no technical difficulties as the chylomicrons are usually less than 1 μ in size. Occasionally aggregations may produce larger globules of fat which tend to vary in size and shape (Swank, 1951). The appearance and uniformity of the particles seen in the counting chamber led us to believe that the figures represented platelet counts. As difficulties were encountered on smearing lipaemic blood, it was not possible to check the results with indirect platelet counts.

A saline extract of acetone dried brain was used for the prothrombin times, and a chloroform extract (Bell and Alton, 1954) in the tests for thromboplastin generation.

RESULTS

As differences were noted as early as 14 days on the experimental diet, the data for the whole period have been pooled. The results presented in Table I and Figs. 1 and 2 can be summarized as follows.

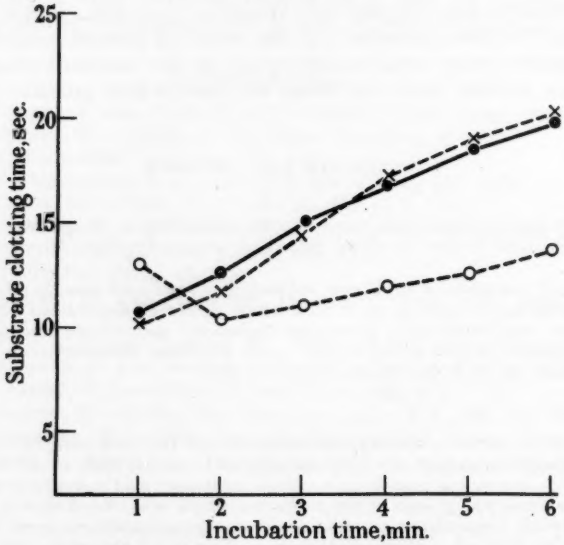

FIG. 1.—Thromboplastin generation test—mean curves.

●——● Atherogenic group III.
×- - - - - - × Thrombogenic group II.
○- - - - - - ○ Control group I.

Differences between experimental groups II and III and the control group I

Shortened coagulation times were noted in groups II and III in the stypven time, the one stage prothrombin time, and the two stage plasma and serum prothrombin times.

Lengthened coagulation times were noted in groups II and III in the last 4–5 min. of the thromboplastin generation test ($P < 0{\cdot}001$) and the thrombo-

TABLE I.—*Blood Coagulation of Normal Rats and of Those Given Diets which Produce Thrombosis and Atherosclerosis*

Group	Diet	Pathological effects produced	Whole blood clotting time		Calcium clotting time (sec.)	Skypven time (sec.)	One-stage prothrombin (sec.)	Two-stage prothrombin time		Platelets (1000)
			Glass (min.)	Silicone (min.)				Serum (sec.)	Plasma (sec.)	
I	Control (10 per cent arachis oil)	None	2·5±0·1	4·5±0·3	64·2±9·2	12·8±0·8	23·2±0·6	341±53	14·80±3·69	728±38
II	40 per cent butter*	Thrombosis	2·8±0·3	6·5±0·4	66·0±16·4	8·9±0·4	21·5±0·4	38·3±7·9	9·27±1·76	1315±105
III	40 per cent arachis oil*	Atherosclerosis	2·7±0·1	4·9±0·5	65·6±7·6	8·3±0·3	21·4±0·8	67·8±10·6	9·73±2·53	920±78
Difference between	Group I and II		NS	C	NS	C	A	C	B	C
	Group I and III		NS	NS	NS	C	A	C	B	A
	Group III and II		NS	A	NS	NS	NS	A	NS	A

* Including cholesterol, cholic acid and thiouracil.
NS (not significant); A, $P < 0{\cdot}05$; B, $P < 0{\cdot}01$; C, $P < 0{\cdot}001$.

plastin screening test ($P < 0{\cdot}05$) suggesting a lack of sustained thromboplastin formation. The silicone clotting times were lengthened in the thrombogenic group only.

Platelet counts were elevated in groups II and III.

An attempt was made to establish whether the defect in the serum prothrombin time could be attributed to abnormal platelet activity. Normal washed platelets were added to whole blood before coagulation took place and the prothrombin time estimated on the serum. The results (Table I) were inconclusive as 2 abnormal samples were corrected completely but in the other 2, only a small correction occurred.

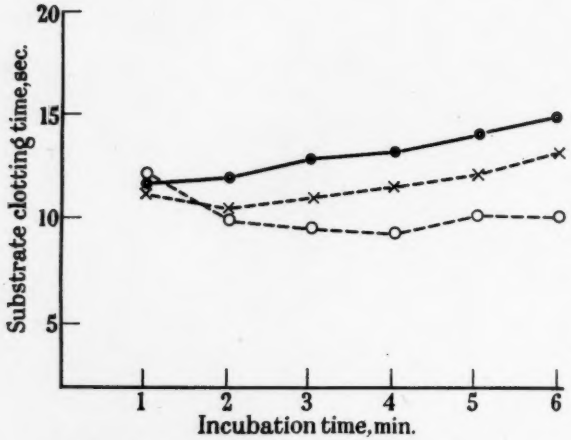

FIG. 2.—Thromboplastin screening test—mean curves.

●——● Atherogenic group III.
×- - - - -× Thrombogenic group II.
○- - - - -○ Control group I.

TABLE II.—*Serum Prothrombin Time (Sec.). The Effect of Adding Washed Platelets to Whole Blood Before Coagulation*

Control group (I)		Thrombogenic group (II)	
Without platelet addition	With platelet addition	Without platelet addition	With platelet addition
180	180	24	420
300	300	24	55
600	600	110	600
600	600	30	70

Differences between the thrombogenic group II and the atherogenic group III

The silicone clotting times were prolonged only in the thrombogenic group. The abnormal reduction in the serum prothrombin time and elevation of the platelet count was greater in the thrombogenic group.

DISCUSSION

The chief purpose of the present work was to investigate the blood coagulation of rats given diets which cause either thrombosis or atherosclerosis (Gresham and Howard, 1960) and to ascertain if a disturbance in the coagulability of the blood could be correlated with the pathological lesions observed.

In both experimental groups some evidence of hypercoagulability was obtained in the form of shortened times in the one stage prothrombin time, the two stage plasma prothrombin time and the stypven time. Other results suggest hypocoagulability. Thus the times were lengthened in the last 4–5 min. of the thromboplastin generation and thromboplastin screening tests, the two stage serum prothrombin times were shortened, and the silicone clotting time lengthened. The interpretation of such a complex abnormality is difficult.

This coagulation defect is similar to that reported in rabbits fed cholesterol (Merskey, 1957). Abnormal prothrombin consumption and thromboplastin generation were noted. While the one stage prothrombin time was normal, the assays for factor VII and prothrombin were elevated. Merskey was unable to reach any definite conclusions regarding the precise nature and significance of the coagulation abnormality. In our experiments, preliminary correction tests of the abnormal serum prothrombin suggested possible abnormality in platelet activity.

The shortened stypven times in the experimental rats resembles the defect seen in idiopathic hyperlipaemia in man (Nitzberg, Peyman, Goldstein and Proger, 1959). In this condition the degree of abnormality is striking compared with the slight but consistent shortening of the stypven times seen in alimentary lipaemia (Fullerton, Davie and Anastasopoulos, 1953; O'Brien, 1955).

In the experimental animals no differences were noted in the stypven time between the thrombogenic and atherogenic group. This is consistent with the view that this test is a rough index of lipaemia (Mandel, Mermall, Preston and Silverman, 1958) and reflects only the " platelet like " activity of lipaemic plasma (O'Brien, 1960).

The platelet counts were elevated to a greater extent in the thrombogenic group. As differences occurred as early as 2 weeks, when no microscopic cardiovascular lesions were present, it is unlikely that the thrombocytosis is a result of the vascular lesions. The relationship between thrombo-embolic disease and elevated platelet counts is well established in the post-operative and post-partum periods as well as in the thrombocythaemic states (Moolten, Vroman, Vroman and Goodman, 1949 ; Wright, 1952). In the pathogenesis of ischaemic heart disease only increased platelet stickiness has been implicated (McDonald and Edgill, 1958) but no studies of this factor were carried out in the present series. Reports of the effect of lipaemia on platelet activity are conflicting. A diminution of the platelet count is reported during alimentary lipaemia (Mustard, 1957 ; Swank, 1951). The older literature (Moolten *et al.*, 1949) has several reports of elevated platelet counts and increased platelet adhesiveness after fat feeding but no significant abnormalities are reported in idiopathic hyperlipaemia.

The fibrinolytic activity of rats given a similar thrombogenic diet has been studied by Tillman, O'Neal, Thomas and Hixon (1960) using a thromboelastograph. Decreased fibrinolysis was found in the rats given a butter diet (thrombogenic)

compared with corn oil (atherogenic). The authors recommend this method for studying the thrombogenic propensities of various diets.

More data are needed to establish the value of differences noted between the thrombogenic and atherogenic groups in the serum prothrombin time, silicone clotting time and platelet counts.

SUMMARY

Rats were given diets known to produce either thrombosis or atherosclerosis. Coagulation studies compared with a normal control group showed no differences in the whole blood glass clotting time and the calcium clotting time. In both experimental groups the stypven times, one stage prothrombin time, two stage serum and plasma prothrombin times were decreased. Platelet counts were elevated and times in the thromboplastin generation test were prolonged.

Differences were noted between the atherogenic and thrombogenic diets; in the thrombogenic group the platelet count was higher, the serum prothrombin time lower and the silicone clotting time increased.

We are grateful for the technical assistance of Miss S. Tomlin.

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THE ZERO EFFECT DOSE (E_0) AS AN ABSOLUTE NUMERICAL INDEX OF ANTIVIRAL CHEMOTHERAPEUTIC ACTIVITY IN THE POX VIRUS GROUP

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In previous work on the antiviral activity of isatin β-thiosemicarbazone and its derivatives in mice infected intracerebrally with vaccinia virus (Bauer and Sadler, 1960), it was found that the mean reciprocal survival time of mice infected with a constant dose of virus had a linear regression on the logarithm of the dose of the antiviral agent administered. The dose-response line thus obtained could be used as the basis of a conventional 4-point assay which enabled the antiviral activity of a number of derivatives to be compared against that of isatin β-thiosemicarbazone, which was chosen as a standard. This method is very convenient for comparing the activity of different compounds against the same virus, but cannot be used for comparing the activity of the same compound against different viruses. However, further study of the dose-response lines of the vaccinia-isatin β-thiosemicarbazone system has shown that they possess a property which enables the activity of a given compound against vaccinia virus to be measured in absolute numerical terms. The same has been found to hold for other members of the pox virus group, and it has thus been possible by this means to determine the absolute activity of the same compound against a number of different viruses, and to classify certain members of the pox virus group in the order of their sensitivity to the same chemotherapeutic agent.

MATERIALS AND METHODS

Viruses

The viruses were used in the form of a 10 per cent suspension of infected mouse brain in glycerol. *Neurovaccinia*—IHD strain, as used in previous work (Bauer, 1955); WR strain; Williamsport strain (Minton, 1951). *Rabbitpox*—Utrecht strain, adapted to passage in mouse brain (Bauer and Sheffield, 1959). *Cowpox*—Brighton strain, obtained from Prof. A. W. Downie, and adapted to passage in mouse brain. *White cowpox*—a variant of the preceding virus forming white pocks, obtained from the same source and adapted to passage in mouse brain. *Ectromelia*—Sandom strain (pseudolymphocytic choriomeningitis).

Treatment

Isatin β-thiosemicarbazone was injected subcutaneously in a volume of 0·1 ml. in a dose ranging in different experiments from 0·005 mg. to 20 mg. The compound was given twice a day, beginning a few hours after infection and continuing for 4–5 days, and was made up in a diluent consisting of 17·5 per cent aqueous ethanol containing 0·75 per cent gum arabic. High doses were prepared by grinding the compound in a mortar and suspending it in the diluent. Low doses were prepared by dissolving a selected weight of compound in ethanol under reflux and then adding diluent to give the required concentration: further serial 2-fold dilutions were then prepared as required. Control mice were injected subcutaneously with 0·1 ml. of diluent at the same times as the treated mice.

Determination of dose-response line

Six groups of 6 mice were infected with virus intracerebrally. The first and last groups were used as controls ; the remaining 4 groups were treated with isatin β-thiosemicarbazone in 4 dose levels increasing in a constant ratio of 2. The dose levels selected depended on the sensitivity of the virus being investigated, and will be given in the experimental section. The animals were observed for 14 days and the survival times were recorded in half-days and converted to reciprocals ; animals which showed no symptoms over a period of 14 days were considered to have survived indefinitely, and were assigned a reciprocal survival time of zero. The mean reciprocal survival time was calculated for each group and plotted as ordinate against the dose of compound on a $\log_2$ scale. The dose-response line was then fitted to the data by the method of least squares.

RESULTS

Dose-response lines of the action of isatin β-thiosemicarbazone against various pox viruses

Typical dose-response lines of the antiviral action of isatin β-thiosemicarbazone against rabbitpox, the IHD strain of neurovaccinia, white cowpox and cowpox viruses are shown in Fig. 1. The WR and Williamsport strains of neurovaccinia gave lines similar to that of the IHD strain, and are therefore not included in Fig. 1. The lines all have a similar slope ; a given increment of dose therefore produces a similar increment in antiviral effect, and this implies that the compound is acting against the different viruses in the same way. The range of doses covered by each line varies over a wide range, from 0·005–0·08 mg. for rabbitpox, to 2·5–20 mg. for cowpox. The viruses therefore differ in their susceptibility to the chemotherapeutic effect of the compound, which clearly has a high activity against rabbitpox and low activity against cowpox. It was not possible to demonstrate any chemotherapeutic effect of isatin β-thiosemicarbazone against ectromelia infection in mice, although a slight but definite effect can be shown in tissue culture (Sheffield, Bauer and Stephenson, 1960) ; no dose-response line can therefore be presented.

It can be further seen from Fig. 1 that each dose-response line has 2 parameters which could be used for a quantitative evaluation of the chemotherapeutic activity of the compound, the abscissa intercept and the value of the abscissa at an ordinate corresponding to the mean survival time in the absence of treatment. The first represents the dose of compound which produces the maximum effect and protects all the mice against specific death, and the second represents the dose which produces no effect. It should be particularly noted that there is no 50 per cent point which is a natural parameter, since the ordinate corresponding to 50 per cent survival will naturally vary in accordance with the days on which the deaths occurred. The dose which produces no effect, which will be referred to as the zero effect dose, E_0, would be a convenient quantitative measure of antiviral chemotherapeutic effect, provided that it is reproducible within acceptable limits of experimental variation and also independent of the dose of virus used in determining it.

Relation between E_0 of isatin β-thiosemicarbazone and dose of neurovaccinia virus

In Fig. 2 dose-response curves for isatin β-thiosemicarbazone are presented which were obtained in 4 separate experiments in which various doses of neurovaccinia virus were used. The individual values of E_0 are given by the abscissae

of the points of intersection of the curves with the corresponding control ordinates, and were calculated from the relation $\log E_0 = \log \bar{x} + b^{-1}(t_c^{-1} - \bar{t}^{-1})$, where $\bar{x}$ is the mean dose, b the regression coefficient of the compound dose-response

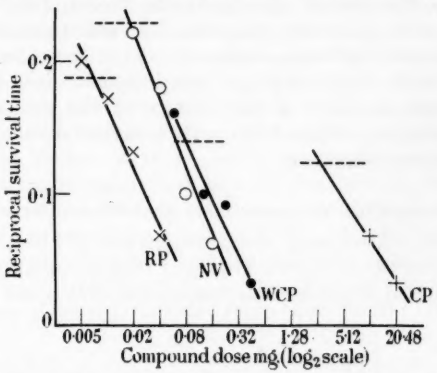

FIG. 1.—Dose-response lines of the antiviral action of isatin β-thiosemicarbazone against 4 pox viruses. *Ordinate*; reciprocal survival tine: *abscissa*; dose of compound on $\log_2$ scale. RP—rabbitpox; NV—neurovaccinia; WCP—white cowpox; CP—cowpox. The dotted lines indicate the mean values of the ordinates obtained in the absence of treatment.

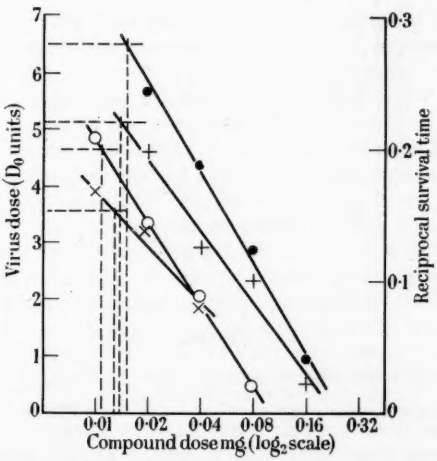

FIG. 2.—Dose-response lines of the antiviral action of isatin β-thiosemicarbazone against varying doses of neurovaccinia virus. *Ordinates*; left—$\log_{10}$ dose of virus in D_0 units, right—reciprocal survival time: *abscissa*; dose of compound on $\log_2$ scale. The dotted lines indicate the mean values of the ordinates obtained in the absence of treatment.

line, t_c^{-1} the control ordinate and $\bar{t}^{-1}$ the mean test ordinate. The value of the control ordinate is related to the log dose of virus used for infection expressed in D_0 units, which may be obtained by dividing the ordinate by 0·0436, the mean value of the regression coefficient of the neurovaccinia virus dose-response curve (Bauer, 1960). The log dose can be converted to conventional LD_{50} units by sub-

tracting 1·6 log unit. The calculated values for E_0 were 0·0128, 0·0107, 0·0142 and 0·0146 mg., corresponding to log virus doses of 3·56, 4·70, 5·07 and 6·41 D_0 units (1·96, 3·10, 3·47 and 4·81 log LD_{50}). The E_0 thus varies over a range of less than 2-fold when the dose of virus is varied over a range of nearly 1000–fold, and the regression of E_0 upon the reciprocal survival time showed no significant deviation from parallelism with the ordinate. E_0 is therefore invariant and independent of dose of virus under constant conditions of dosing, and can therefore be used as an absolute measure of the activity of the compound against neurovaccinia virus without any comparison with an arbitrary standard of activity in the form of a reference compound.

The E_0 of isatin β-thiosemicarbazone in other pox virus infections

The E_0 could also be used as an absolute measure of chemotherapeutic activity in other pox virus infections as well, provided that it remained invariant for each virus under the standard experimental conditions, and could therefore be used to

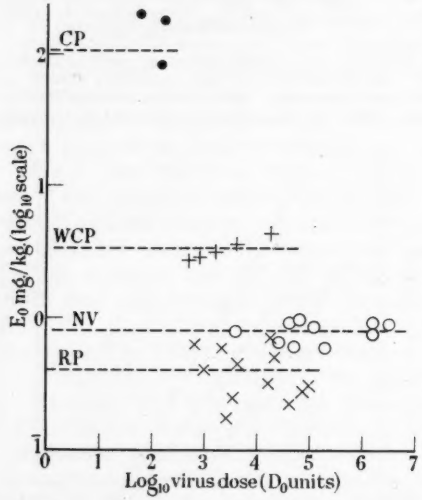

FIG. 3.—Values of E_0 for 4 different pox viruses. *Ordinate*; E_0 in mg. per kg. on $\log_{10}$ scale: *abscissa*; $\log_{10}$ dose of virus used for infection in D_0 units. RP—rabbitpox; NV—neurovaccinia; WCP—white cowpox; CP—cowpox. The dotted lines indicate the respective mean values of E_0.

characterize the antiviral activity of isatin β-thiosemicarbazone against other viruses than vaccinia. This possibility was investigated in a series of experiments similar to those of Fig. 2, carried out with rabbitpox, white cowpox and cowpox viruses. Dose-response curves of antiviral activity were obtained with each virus over a range of virus doses; E_0 was calculated in each case, and converted to mg. per kg. of body weight. The results obtained are shown in Fig. 3, in which the values of E_0 are plotted against log virus titre in D_0 units. The results for neurovaccinia shown in Fig. 2 are included together with the results of 9 other determinations. In the case of each virus the coefficient of regression of E_0

against log virus dose was calculated, and was found not to differ significantly from zero. The E_0 was therefore invariant for each virus over the range of virus doses used in the experiments, the extent of the range being limited in practice by the maximum virus titres attainable in the preparation of mouse brain used for infection. The mean value of E_0 was calculated for each virus, and is represented in Fig. 3 by a line of zero slope drawn through the corresponding points. It will be noted that E_0 is different for each virus, the value for rabbitpox being $0{\cdot}41 \pm 0{\cdot}17$ mg./kg., for neurovaccinia $0{\cdot}75 \pm 0{\cdot}18$ mg./kg., for white cowpox $3{\cdot}34 \pm 0{\cdot}54$ mg./kg. and for cowpox $159{\cdot}7 \pm 54{\cdot}4$ mg./kg. The differences between the adjacent means were in all cases highly significant ($P < 0{\cdot}001$). The four pox viruses are therefore not equally sensitive to the chemotherapeutic effect of isatin β-thiosemicarbazone, and in this respect form a sequence in accordance with the corresponding values of E_0, which thus enable the differences in sensitivity observed in the experiments of Fig. 1 to be expressed precisely in numerical terms. The values of E_0 for the Williamsport and WR strains of vaccinia virus did not differ significantly from that for the IHD strain of vaccinia virus.

DISCUSSION

The E_0, in addition to being a useful measure for putting antiviral chemotherapy upon a firm quantitative basis, has certain implications of theoretical interest.

It is generally assumed that since viruses presumably use cell systems for their replication (although there is no evidence for this), successful antiviral chemotherapy requires the discovery of some substrate, enzyme or other biochemical target which is more important for the virus than it is for the host cell. This concept has led to a search for antimetabolites, unnatural purines and amino acids, competitive and non-competitive inhibitors, compounds which will react with nucleic acids, and other empirical gambits of contemporary chemotherapeutic strategy. Such an approach may be justified in the cases of antibacterial and antitumour chemotherapy, but the results obtained with the pox diseases suggest very strongly that this approach is misguided in the case of the chemotherapy of virus infections. Vaccinia and ectromelia viruses growing in the same tissue, in mouse brain, presumably utilize the same cell systems in their replication, yet one virus is highly sensitive to chemotherapy and the other is scarcely sensitive at all. It is not beyond the bounds of possibility that a system affected by isatin β-thiosemicarbazone is necessary for the multiplication of vaccinia virus and that ectromelia is able to dispense with it, but this hypothesis, although compatible with an all-or-none occurrence of sensitivity, cannot easily explain the gradation in sensitivity disclosed by the values of E_0. The most reasonable explanation of the experimental findings is that the compound is acting against the virus and not against the cell. This implies that the compound is complementary in its chemical structure and configuration to some part of the virus particle, the degree of complementariness varying in accordance with the changes in structural detail on the molecular level which may be expected to occur between one virus and another. A necessary corollary of this theory is that the compound might be made active against viruses which are unaffected by it by making appropriate changes in its structure in order to improve the goodness of fit against the sensitive regions of the virus material.

The E_0 is also of significance in virus genetics. A number of marker characters have been observed in the pox viruses and attempts have been made to study their behaviour in reactivation experiments and to interpret the results along conventional genetic lines. It is always assumed in this type of work that a character is either present or absent, *i.e.* the virus is either heat sensitive or stable, the pocks are either red or white (*i.e.* red or not red), and so on. The presence or absence of the character is then ascribed to the presence or absence of a hypothetical gene. Chemotherapeutic sensitivity is clearly a marker character in the pox viruses, but the values observed for E_0 shows that it is not distributed in an all-or-none manner. It would thus be entirely unjustifiable to describe rabbitpox and ectromelia as *S* and *s* respectively (sensitive and insensitive) along conventional genetic lines, since the values obtained for the E_0 of the various pox viruses clearly show that chemotherapeutic sensitivity is distributed continuously, and thus cannot be due to the presence or absence of a single gene. It is quite possible that a similar continuous distribution would be found if the other marker characters were determined in absolute numerical values. If sensitivity to thermal inactivation, for example, were expressed as the energy of activation of the process instead of as the presence or absence of an effect under arbitrarily selected experimental conditions, it is quite possible that a continuous distribution would be found for this marker character as well, and before erecting a structure for the genetics of the pox viruses it might be as well to approach the problem anew with rigorously quantitative experimental techniques.

SUMMARY

The response of mice infected with neurovaccinia virus to treatment with isatin β-thiosemicarbazone bears a linear relation to the logarithm of the dose of compound administered. The point of intersection of this dose-response line with the value of the response obtained in untreated mice represents the dose at which no chemotherapeutic effect is obtained. This value is termed the zero effect dose, or E_0, and was found to be independent of the dose of virus used for infection. The value of E_0 therefore characterizes the chemotherapeutic effect of isatin β-thiosemicarbazone in absolute numerical terms without relation to an arbitrary standard of activity. Dose-response curves of antiviral effect were also obtained with rabbitpox, cowpox and white cowpox viruses, and were similarly independent of the virus dose. The values of E_0 thus obtained enabled a comparison to be made of the chemotherapeutic activity of the same compound against different viruses. The order of sensitivity of the viruses was as follows: ectromelia $\ll$ cowpox $<$ white cowpox $<$ neurovaccinia $<$ rabbitpox. The implications of the concept of the zero effect dose upon the planning of antiviral chemotherapy and upon virus genetics are pointed out.

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GLYCOGENOLYTIC EFFECT OF 2-MERCAPTO-ETHYLAMINE IN EPIPHYSEAL CARTILAGE*

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MARKED depletion of liver glycogen in mice and rats has been induced by the administration of the sulphhydryl compound 2-mercapto-ethylamine, $HSCH_2CH_2NH_2$ (cysteamine), or the disulphide form (cystamine) (Bacq, Herve and Fischer, 1953*a*; Sokal, Sarcione and Gerszi, 1959). As this glycogenolytic action was also found to occur in adrenomedullated and in pancreatectomized rats, a direct effect on liver glycogen has been postulated (Sokal *et al.*, 1959).

2-mercapto-ethylamine is not only a radiation protective agent, (Bacq *et al.*, 1953*b*) but it has also been shown to be a potent osteolathyrogenic agent (Dasler and Milliser, 1958; Ramamurti and Taylor, 1958). We have previously shown that the lesion of acute experimental osteolathyrism induced in the epiphyseal cartilages of weanling rats by the administration of the known lathyrogenic agents, amino-acetonitrile and methylene-amino-acetonitrile, was characterized by a marked depletion of glycogen in the cells of the epiphyseal plate (Karnovsky, 1960). Furthermore, the cells of lathyric epiphyseal cartilage had low or absent phosphorylase activity, when compared with normal control cartilage, as shown by their decreased ability to synthesize glycogen, given glucose-1-phosphate as substrate. These changes were demonstrated by histochemical techniques. The decrease in the amount of glycogen and in phosphorylase activity occurred independently of reduced dietary intake and failure to gain weight, and was also readily obtained in the adrenalectomized animal.

This communication reports that 2-mercapto-ethylamine, administered by mouth to weanling male rats, has a similar, apparently direct, glycogenolytic effect in the cells of epiphyseal cartilage. The glycogenolytic action of 2-mercapto-ethylamine may be detected before the histopathological changes characteristic of the lathyric lesion are readily detectable in the growth cartilage.

MATERIALS AND METHODS

Male rats, 23 days old, of the Sprague-Dawley strain, weighing 43–57 g. (mean 52 g.) were used in the experiments on intact animals. In the experiments on adrenalectomized rats, 27 day old rats, weighing 53–60 g. (mean 51 g.) and adrenalectomized 3 days previously, were used. β-mercapto-ethylamine (California Foundation for Biochemical Research, Lot No. 4451), supplied as the hydrochloride, was dissolved in water and administered by stomach tube, in the doses and for the periods of time indicated in Tables I and II. Control rats received an equivalent amount of water by stomach tube. The rats were fed on a standard laboratory diet of Purina Rat Chow, and each control rat was paired by weight gain or loss to its experimental partner; *i.e.* the daily food intake of the controls was adjusted so that

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the control rat of each pair lost or gained weight at the same rate as its experimental partner. The intact rats were allowed tap water for drinking *ad libitum*; the adrenalectomized rats were maintained on drinking water containing 1 per cent sodium chloride.

The proximal tibial epiphyseal cartilage was chosen for evaluation. The proximal ends of the tibiae were fixed in ice-cold 10 per cent formol alcohol for 12 hr. The bones were double embedded in celloidin-Tissuemat[R] (Fisher) without prior decalcification, and sections were cut at a thickness of 6 μ. Glycogen was demonstrated in celloidin-coated sections by the periodic acid-Schiff technique, without the use of a reducing rinse (McManus and Hoch-Ligeti, 1952). Saliva and diastase treated controls were used to confirm the presence of glycogen. The combined periodic acid-Schiff-Alcian blue technique (Mowry, 1956) was used on duplicate sections to correlate changes in the acid mucopolysaccharides of the cartilage matrix with changes in the amount and distribution of glycogen.

RESULTS

The results obtained in non-adrenalectomized rats are shown in Table I. Daily doses of less than 400 mg./kg. body weight of 2-mercapto-ethylamine did not lead to any change in epiphyseal cartilage, even when the lathyrogen was administered for a period of 8 days. Daily doses of 2-mercapto-ethylamine of 400 mg./kg., or more, led to the virtual disappearance of glycogen from the cells of the epiphyseal cartilage. A dose level of 400 mg./kg. body weight of 2-mercapto-ethylamine given for 4 days led to the development of the histopathological changes characteristic of osteolathyrism, and glycogen was markedly depleted in the cartilage cells (Fig. 1). With doses of 600 mg./kg. body weight daily, or more, given for 1 or 2 days, glycogen markedly decreased in or disappeared from the cells before the histopathological changes of osteolathyrism in growth cartilage could readily be detected (Fig. 3). The slight changes noted at this stage consisted of a variable increase in the staining intensity with Alcian blue of the matrix between the cell columns, and slight and irregular increase in the width of the plate. The complete disorganization of the cartilage as seen in Fig. 1 occurs at a later stage.

With doses above 600 mg/kg. body weight daily some rats succumbed. These rats also showed absence of glycogen in the cartilage cells, but were not used in assessing the results (except in Table II, C). However, the disappearance of glycogen was clearly not a post mortem change.

EXPLANATION OF PLATE

All figures are of the proximal tibial epiphysis, stained with P.A.S.-Alcian blue. All magnifications × 94.

FIG. 1.—Lathyric rat, treated with 2-mercapto-ethylamine 400 mg./kg. daily for 4 days. The cell columns are greatly disorganized and the cells are embedded in a copious matrix. The majority of the cells are lacking in glycogen. There is a split containing bone marrow cells in the middle of the cartilage.

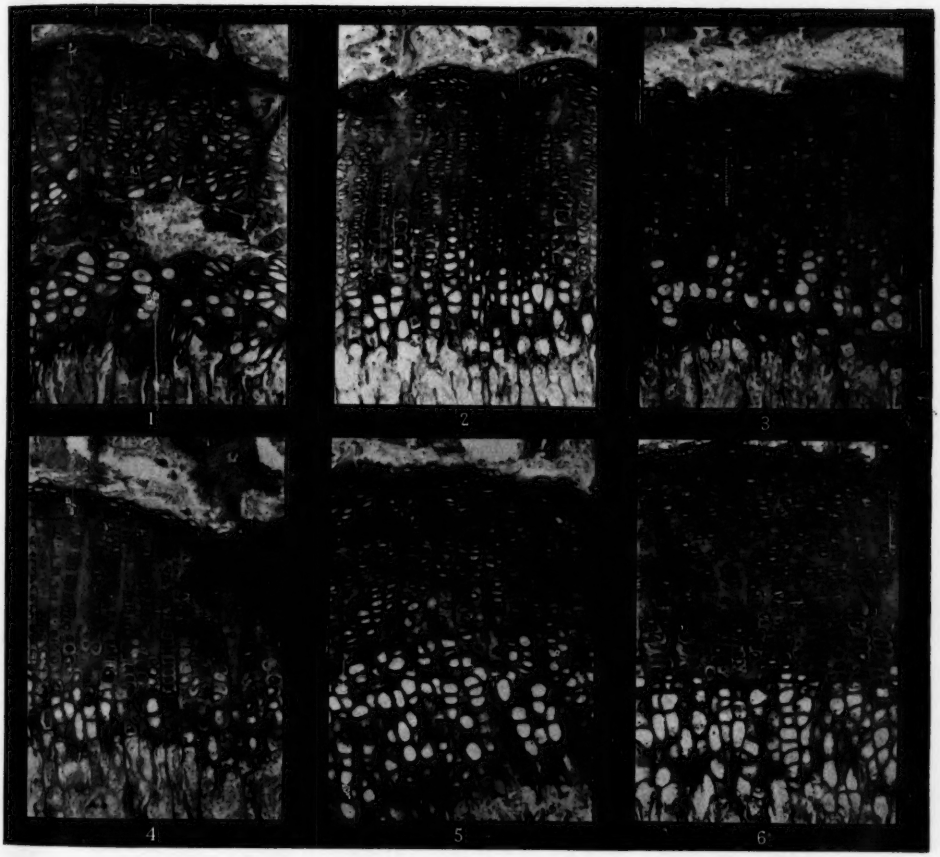
FIG. 2.—Control rat, paired by weight gain to lathyric rat of Fig. 1. The cells of the maturing and hypertrophic zones contain considerable amounts of glycogen.

FIG. 3.—Lathyric rat, treated with 2-mercapto-ethylamine 800 mg./kg. daily for 2 days. The cells are devoid of glycogen. The intercellular matrix shows increased staining with Alcian-blue, but otherwise the characteristic changes of lathyrism are absent. (cf. Fig. 1.)

FIG. 4.—Control rat, paired by weight loss to lathyric rat of Fig. 3. The plate is narrowed due to cessation of growth (cf. Fig. 2) but glycogen is present in considerable amount.

FIG. 5.—Lathyric rat, adrenalectomized, treated with 2-mercapto-ethylamine 400 mg./kg. for 2 days. Appearance similar to Fig. 3, except that plate is irregularly widened.

FIG. 6.—Control rat, adrenalectomized, paired by weight gain to lathyric rat of Fig. 5. Glycogen is present in considerable amount.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The depletion of glycogen was not due to failure to gain weight as the control rats, paired by weight to their experimental partners, had considerable amounts of glycogen in their epiphyseal cartilages despite the restriction in daily food intake imposed on them, and their consequent lack of growth.

Adrenalectomized rats (Table II) were more sensitive than intact rats to 2-mercapto-ethylamine, and glycogen depletion was observed with lower doses. The mortality with high doses was greater as compared with non-adrenalectomized rats. The decrease in glycogen was clearly detectable before the lesion of lathyrism had fully developed (Fig. 5). The increased sensitivity of adrenalectomized rats

TABLE I.—*Effect of Cysteamine on Epiphyseal Cartilage Glycogen in Non-adrenalectomized Rats*

	Daily dose of cysteamine (mg./kg. body weight)	Number of days cysteamine given	Number of rats	Glycogen in EC*	Degree of lathyric changes in EC†	Mortality	Weight change	
							Initial weight	Final weight
A. Exper.	200	4	4	++	0	0	50 ± 1	63 ± 2
Control	—	—	4	++	0	0	46 ± 1	59 ± 3
Exper.	200	8	4	++	0	0	53 ± 1	93 ± 2
Control	—	—	4	++	0	0	52 ± 2	95 ± 2
B. Exper.	400	4	4	±(4)	++	0	53 ± 1	93 ± 2
Control	—	—	4	++	0	0	52 ± 2	95 ± 2
C. Exper.	600	2	5	±(3)	±	1	43± 2	42 ± 1
Control	—	—	5	++	0	0	44 ± 2	41 ± 2
D. Exper.	800	1	4	±(3)	0	1	53 ± 1	51 ± 1
Control	—	—	4	++	0	—	50 ± 2	48 ± 3
Exper.	800	2	5	±(4)	+	1	57 ± 1	51 ± 2
Control	—	—	5	++	0	0	56 ± 1	52 ± 1
Exper.	800	3	4	−(4)	++	0	53 ± 1	44 ± 2
Control	—	—	4	++	0	0	53 ± 1	46 ± 0

*EC—epiphyseal cartilage. 0 = glycogen absent; ± = glycogen markedly decreased or absent; ++ = glycogen present in considerable amount. Figures in parentheses refer to number of rats affected.
†EC—epiphyseal cartilage. 0 = no lathyric changes; ± = minimal changes; + = moderate changes; ++ = marked changes.

TABLE II.—*Effect of Cysteamine in Epiphyseal Cartilage Glycogen in Adrenalectomized Rats*

	Daily doses of cysteamine (mg./kg. body weight)	Number of days cysteamine given	Number of rats	Glycogen in EC*	Degree of lathyric changes in EC†	Mortality	Weight change	
							Initial weight	Final weight
A. Exper.	100	5	4	±(3)	0	1	53 ± 1	56 ± 4
Control	—	—	4	++	0	0	52 ± 2	56 ± 3
B. Exper.	400	2	5	±(4)	±	1	59 ± 1	61 ± 2
Control	—	—	5	++	0	0	55 ± 2	62 ± 1
C. Exper.	600	2	5	0(5)	0++	5	60 ± 3	54 ± 2
Control	—	—	5	++	0	0	57 ± 2	56 ± 2

*EC—epiphyseal cartilage. 0 = glycogen absent; ± = glycogen markedly decreased or absent; ++ = glycogen present in considerable amount. Figures in parentheses refer to number of rats affected.
†EC—epiphyseal cartilage. 0 = no lathyric changes; ± = minimal changes; ++ = marked changes.

to lathyrogenic agents has previously been noted (Karnovsky, 1960), and corticoids partially protect against the development of the cartilaginous lesions of experimental lathyrism (Selye and Bois, 1957).

In both intact and adrenalectomized lathyric rats it was evident that the articular cartilages were singularly unaffected, an observation which has also been made in regard to osteolathyrism induced by amino-acetonitrile and methylene-amino-acetonitrile (Karnovsky, 1960).

DISCUSSION

The oral dose of 2-mercapto-ethylamine required to produce clear-cut hepatic glycogenolysis in intact rats was 200–500 mg./kg. (Sokal *et al.*, 1959) and the effect was seen within a few hours after a single dose. In the case of epiphyseal cartilage glycogen, a dose of 200 mg./kg. daily for 8 days had no clear-cut effect, and higher daily doses given over a period of one to several days, were required. This discrepancy may be due, in part, to the qualitative nature of the histochemical technique used for detecting changes in glycogen levels : only marked differences between experimental and control rats could readily be assessed. On the other hand, epiphyseal cartilage may be relatively insensitive to 2-mercapto-ethylamine as compared with liver. Further, 2-mercapto-ethylamine is rapidly metabolized or excreted (Bacq *et al.*, 1953*b*) so that a single daily dose would probably not maintain high blood levels.

The hepatic glycogenolytic action of 2-mercapto-ethylamine was found to occur in both adrenomedullated and in pancreatectomized rats, and therefore, a direct effect on liver glycogen was postulated (Sokal *et al.*, 1959). In addition, the secretion of adrenaline and of insulin was stimulated in the intact animal. 2-mercapto-ethylamine was also considered to act independently of glycogen. A significant glycogenolytic effect obtained on muscle glycogen was attributed to stimulation of adrenaline secretion.

The effect of 2-mercapto-ethylamine in pancreatectomized rats in regard to epiphyseal cartilage glycogen has not been studied. It is unlikely, however, that insulin would have a glycogenolytic effect peripherally, *i.e.* on epiphyseal cartilage.

In contrast to the situation in muscle, we obtained glycogenolysis in epiphyseal cartilage in adrenalectomized rats, which showed, moreover, increased sensitivity to 2-mercapto-ethylamine. Therefore, the glycogenolytic effect of 2-mercapto-ethylamine on epiphyseal cartilage glycogen is considered to be independent of adrenaline secretion.

The osteolathyrogenic agent, 2-mercapto-ethylamine, has a marked glycogenolytic effect on epiphyseal cartilage glycogen, similar to that previously obtained with the lathyrogenic agents amino-acetonitrile, and methylene-amino-acetonitrile (Karnovsky, 1960). This effect is apparently direct in nature, and may well be similar to the direct glycogenolytic effect obtained in liver. The mode of action whereby 2-mercapto-ethylamine induces glycogenolysis in liver or epiphyseal cartilage is at present unknown.

Lathyrogenic agents induce severe changes in collagen, and these changes have been correlated with some features of the disease, such as increased fragility of the connective tisues (Levene and Cross, 1959). In work published elsewhere (Karnovsky and Karnovsky, 1961) we showed that lathyrogenic agents had profound effects on cartilage metabolism (especially that of sulphated acid

mucopolysaccharides), both *in vivo* and *in vitro*. The relationship of these metabolic changes, and the glycogenolysis described above, to the changes occurring in collagen and the development of the characteristic lesions, is at present a matter of speculation.

SUMMARY

2-mercapto-ethylamine, a known lathyrogenic agent, has a marked and apparently direct glycogenolytic action in the cells of epiphyseal cartilage. The decrease in glycogen content of the cells can be detected before overt histopathological signs of osteolathyrism develop. Glycogen in articular cartilage is unaffected.

The glycogenolytic effect of 2-mercapto-ethylamine is not due to cessation of growth or loss of weight, and is readily obtained in the adrenalectomized rat. Adrenalectomized rats are more sensitive to the lathyrogen,

2-mercapto-ethylamine, and the lathyrogens methylene-amino-acetonitrile and amino-acetonitrile, have similar glycogenolytic effects in epiphyseal cartilage.

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THE MECHANISM OF INHIBITION OF ACUTE X-RAY DAMAGE TO GUINEA-PIG SKIN BY CORTISONE

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NECROSIS of small areas of guinea-pig skin due to localised X-irradiation may be modified and delayed for long periods by treatment with parenteral cortisone (Houghton, Walter and Jones, 1954). As a preliminary to the investigation of the mechanism of this action of cortisone, a detailed study was made of the naked eye and microscopical changes in guinea-pig skin following localised X-irradiation without further treatment. This showed 2 phases of ulceration (Mellett, Walter and Houghton, 1960), designated "superficial" (affecting epidermis only and occurring from the 18th day after irradiation) and "deep" (affecting all levels and commencing between the 26th and 48th day after irradiation). Deep ulceration is associated with vascular degeneration. Healing of the irradiated area may occur between these phases in some animals and, during this interval, there is hyperplasia of the epidermis over the whole irradiated field. Hyperplasia of the hair follicles between the centre and the periphery of the irradiated field gives rise to cyst formation and the production of irregular crescentic elevations, visible to the naked eye, and described as "rampart" rings. Where deep ulceration follows immediately upon superficial ulceration, it is invariably preceded by hyperplasia of the epidermis at the periphery of the field.

Similar microscopical and macroscopical observations have now been made on irradiated animals, of which some received no further treatment and some received cortisone for 42 days from the day of irradiation. This period of cortisone treatment was chosen because previous investigation had shown that, in the majority of animals, deep ulceration occurred between the 26th and 48th post-irradiation days. It was hoped, therefore, that the histological appearances of several skins just before and during deep ulceration might be compared with the histological appearances of skins of cortisone-treated animals in which ulceration had been delayed by cortisone treatment.

MATERIALS AND METHODS

A total of 27 young adult albino guinea-pigs (average weight 504 g.) were irradiated over a circular field (diameter 2·6 cm.) on both flanks. Their backs had previously been clipped without injury to the skin and they were anaesthetized prior to irradiation with Nembutal (20 mg./kg. body weight) given intraperitoneally. Each received an exposure dose of 5000 r in a single irradiation of 2½ min. duration to each field. Apart from the shorter exposure giving a dose of 5000 r instead of 6000 r the irradiation technique was exactly as described by Mellett *et al.* (1960). The smaller dose of irradiation was selected as extensive pilot work had shown it was sufficient to guarantee deep ulceration in all irradiated animals, which were not given other treatment.

Sixteen animals were treated with 10 mg. cortisone acetate (Roussel) daily, by subcutaneous injection into the abdominal wall. The first injection was given in the evening a few hours following irradiation, and subsequent injections were given each morning until the 42nd day after irradiation.

The irradiated areas were inspected daily and, at intervals of 7 days, life-size drawings were made of the 2 irradiated areas on each of the animals.

On the 30th, 36th and 42nd days, the irradiated areas of one side of 1 or 2 control animals and 4 cortisone-treated animals were completely excised, immediately after drawing. Ether anaesthesia was used. Observations were continued on the non-excised area on the opposite side of each animal so treated. Repeated experiments with bilateral irradiation had shown that developments on the 2 sides were remarkably synchronous. Changes in the non-excised areas were therefore taken to indicate what would have occurred in the excised areas, had they been left intact.

After fixation in 10 per cent formol saline, paraffin sections of the excised tissue were prepared in the usual way and stained with haematoxylin and eosin and PAS.

RESULTS

Macroscopic

During the first 21 days after irradiation, epilation occurred in all animals but, as reported by Houghton *et al.* (1954), hair loss was delayed in cortisone-treated animals and the hair tended to remain adherent until the skin desquamated ; then the hair was lost in patches.

Desquamation occurred in all animals between the 20th and 40th days after irradiation, and some animals of both groups showed wet, superficial ulcers at this stage. (Desquamation and superficial ulceration are regarded as having the same cause, *i.e.* death of epidermal cells, superficial ulceration being a localised loss of the whole depth of the epidermis.)

Deep ulceration occurred in some control animals by the 35th day ; 10 of the 11 control animals showed deep ulceration by the 49th day and all had ulcerated by the 55th day (Fig. 1). No cortisone-treated animals showed deep ulceration before the 50th day (8 days after cessation of cortisone treatment).

One cortisone-treated animal was killed on the 41st day, owing to injury. Of the remaining 15, 11 showed no deep ulceration before the 64th day and, in several, it was much longer delayed and less severe (Fig. 2).

Several control animals passed from superficial ulceration to deep ulceration with no interval ; rampart rings were seen for very short periods between the 2 phases of ulceration in 6 control animals. They were observed in 14 cortisone-treated animals. In 7 they lasted from 20–60 days (Figs. 1 and 2.)

Microscopic

In general the changes seen in the cortisone-treated animals did not differ qualitatively from those seen in the controls. For a given naked eye appearance the microscopic appearances did not differ between the two groups. However, the cortisone-treated group did show a great prolongation of the interval between superficial and deep ulceration. For this reason rampart formation was more marked in this group.

Sections from control animals at the 30th day

The epithelium in the centre of the lesion showed great irregularity and in one specimen there was superficial ulceration. Peripherally the epidermis was

thickened to a depth of 8–10 cells (the normal guinea-pig epidermis is of 2–3 cells thickness only). Squamous metaplasia of underlying hair follicles resulted in the formation of keratin-filled cysts. The underlying connective tissue, both dermal

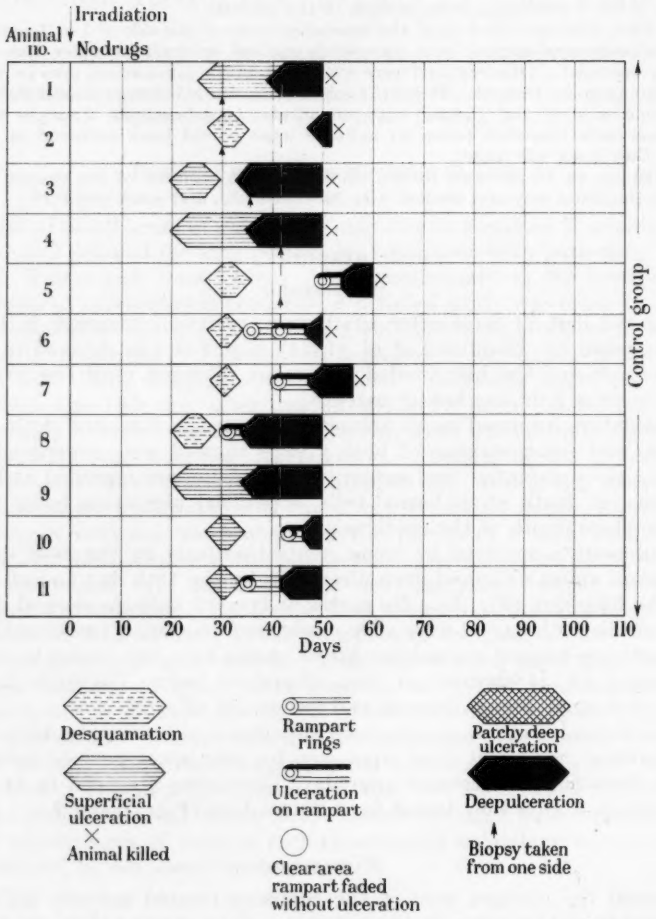
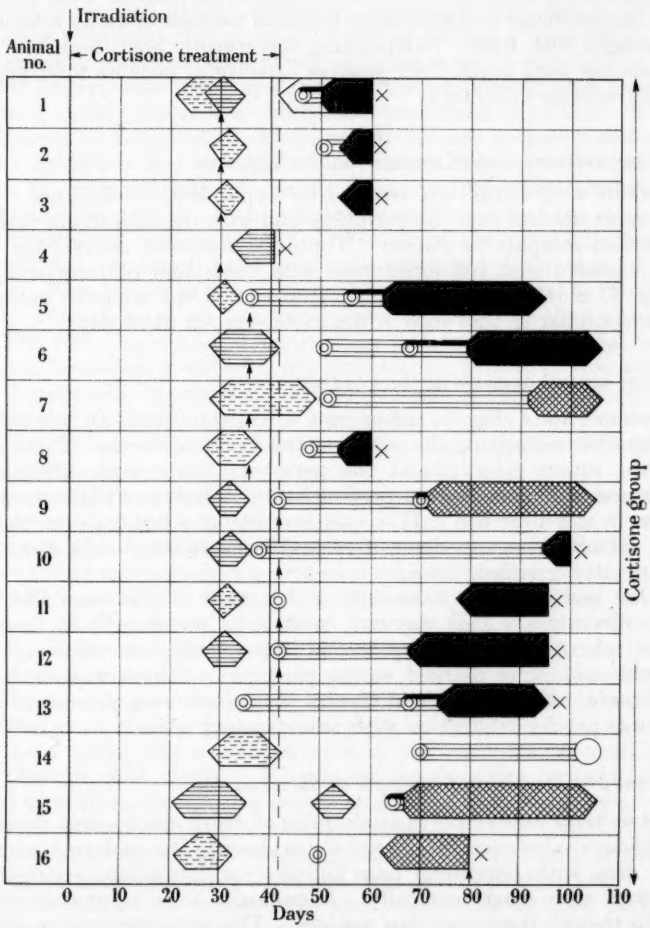


FIG. 1.—Chart showing development of ulceration in control animals.

and subcutaneous, showed enlargement of fibroblasts and scanty infiltration with small round cells and occasional polymorphs. Polymorph infiltration was present in the base of the ulcer. Blood vessels and the underlying muscle appeared normal. These changes have been described previously in greater detail (Mellett *et al.*, 1960).

Sections from cortisone-treated animals at the 30th day

The appearances were similar to those of the control animals. Three of the skins showed small central superficial ulcers although ulceration was noted macro-



For key see Fig. 1.

FIG. 2.—Chart showing development of ulceration in cortisone-treated animals.

scopically in only one of these (No. 1, Fig. 2). The other 2 were described as showing desquamation (Nos. 2 and 3, Fig. 2) but the remaining untouched irradiated areas ulcerated 2 days later (No. 4, Fig. 2). No arteriolar necrosis was seen in any of the specimens.

Section from a control animal at the 36th day

A large deep ulcer was present: the necrosis extended laterally to include the hyperplastic epithelium and the underlying keratin-filled cysts of the ramparts. The dermis, subcutaneous tissues and some muscle were also necrotic. There was haemorrhage and associated fibrinoid necrosis of many arterioles, which stained strongly with PAS. Surrounding the necrotic area there was an inflammatory response with much PAS positive interstitial oedema fluid and a scanty pleomorphic cellular infiltration.

Sections from cortisone-treated animals at the 36th day

Three skins were completely covered by hyperplastic epithelium: the hyperplasia was most marked near the periphery and with the underlying cysts gave rise to well marked rampart formation. The dermis showed many large fibroblasts and scanty small round cell infiltration with occasional polymorphs. One skin (No. 6, Fig. 2) showed a small superficial ulcer and the adjacent epithelium was irregular and similar to that seen in the control group at 30 days.

Sections from control animals at the 42nd day

One section (No. 4, Fig. 1) taken from a control animal at this stage showed a large deep ulcer occupying the centre of the irradiated area. There was hyperplasia of the intact epidermis at the periphery. Abnormal fibroblasts, small round cells and polymorphs were present in the dermis and there were numerous polymorphs in the ulcer bed. Fibrinoid necrosis of arterioles near the ulcer was also seen. Haemorrhage, oedema fluid and inflammatory cells were also noted between underlying muscle fibres.

The other section taken from a control animal at this time (No. 5, Fig. 1) showed no ulceration. This was not, in fact, to occur until 13 days after the section was taken. There was epidermal hyperplasia over the whole irradiated area and this was more marked at the periphery. There was no degeneration of blood vessels. Many abnormal bizarre fibroblasts were present in the dermis and there was patchy infiltration with inflammatory cells.

Sections from cortisone-treated animals at the 42nd day

These 4 sections taken from animals, none of which was to show deep ulceration less than 26 days subsequently, showed hyperplasia of the epidermis over the whole irradiated area with peripheral hair follicle cyst formation, giving rise to the rampart rings seen macroscopically. Micronuclei were commonly seen in epidermal cells though there were few mitoses. The connective tissue showed some enlarged fibroblasts but these were not as bizarre as those seen in control animals. There was very scanty infiltration with inflammatory cells, and no oedema was seen. The underlying muscle was normal and no arteriolar necrosis was noted.

A single section from a cortisone-treated animal (No. 16, Fig. 2) taken on the 80th post-irradiation day, when a deep ulcer had appeared, showed appearances similar to those of the control animal (No. 4, Fig. 1) with a deep ulcer which had developed by the 42nd day. There was extensive necrosis of collagen, muscle and blood vessels.

DISCUSSION

Prior to this investigation several theories had been advanced to explain the prevention of deep (2nd phase) necrosis of guinea-pig skin due to X-irradiation when cortisone treatment was given for a period following irradiation.

One possibility was that, as a direct or indirect result of radiation injury, an uncontrolled hyperplasia of the epidermis of the whole irradiated area occurred. This followed closely upon the short-lived superficial ulceration of epidermis only. Demands on the available blood supply became excessive and cells of the hyperplastic epidermis died through inadequate nutriment. Cortisone treatment was known to slow the rate of mitosis in mouse epidermis (Bullough, 1952; Green and Ghadially, 1951). It was therefore suggested that the second phase of epidermal breakdown accompanied by necrosis of the whole thickness of the dermis could be prevented by a cortisone-controlled limitation of epidermal hyperplasia. This theory is not supported by the histological findings for cortisone-treated animals show no less hyperplasia of epidermis than animals not so treated. The phenomenon of rampart rings due to hyperplasia of hair follicle cells and cyst formation is indeed more prominent. Moreover this state of gross epidermal hyperplasia in cortisone-treated animals is seen to be prolonged for very long periods (up to 60 days). This indicates not only the failure of cortisone to inhibit hyperplasia but also that there is no lack of essential nutriment for epidermal cells in these conditions.

Another possibility was that a florid fibrotic reaction in the dermis followed the initial (possibly direct) radiation injury. This fibrosis led to "strangling" of blood vessels and therefore the dry deep necrosis observed as deep ulceration. It was known that cortisone had a profound inhibitory effect on the formation of fibroblasts and fibrous tissue after various types of injury, especially in the vicinity of wounds, or chemically-induced tissue necrosis (Taubenhaus, 1953; Ragan, Howes, Plotz, Meyer and Blunt, 1949; Taubenhaus and Amromics, 1950).

It was therefore suggested that deep post-irradiation necrosis secondary to "dermal fibrosis" in guinea-pig skin was prevented by this action of cortisone. In fact histological studies have shown that though abnormal fibroblasts are present fibrosis below the irradiated area is not conspicuous until after deep ulceration (Mellett *et al.*, 1960). Fibrosis cannot therefore be held to be causative of deep ulceration, though it is true that delay of deep ulceration by cortisone does delay the ultimate fibrosis consequent upon the deep lesion. Whether the degree of ultimate fibrosis and shrinkage of the affected area is decreased or prevented by cortisone treatment has not been determined as yet.

Cortisone is known to produce lymphocytolysis for a brief period and to suppress lymphocytopoiesis for a long period after a single injection (Dougherty and White, 1945) and further work has shown that involution of lymphoid tissue is maintained for the whole period of any continuous treatment (Dougherty, 1952). Reticulo-endothelial cells, lymphocytes and plasma cells have all been implicated as the principal cellular sites of antibody synthesis (Burnet and Fenner, 1949; Dougherty, 1953). It would therefore seem possible that cortisone treatment could weaken and prevent an "immune response" to radiation-damaged tissue which might otherwise be treated as a homograft and eventually slough.

Previous investigation, however, has failed to reveal any simple immuno-

logical explanation for X-ray damage. If one area of guinea-pig skin is irradiated and, 3 weeks later, another area is irradiated, the second area does not undergo superficial epidermal or deep necrosis sooner after irradiation than the first (Houghton, Nassau, Riches and Walter, 1956). This implies that no circulating antibody at any rate is produced by the presence of one irradiated area, such as to affect the rate of reaction of the whole organism to the presence of the second irradiated area. At present, therefore, there is no evidence that the inhibitory effect of cortisone is mediated by interference with an immune response.

Other hypotheses relate to direct and indirect effects of radiation damage to blood vessels and the possible prevention of those effects by cortisone. Such hypotheses are favoured for several reasons. Macroscopically the deep necrosis of guinea-pig skin which we describe is reminiscent of dry gangrene of the human extremities seen as the result of vascular disease. It also closely resembles the localised necrosis of guinea-pig skin following the local injection of adrenachrome, which gives rise to vasoconstriction. The present findings are that degeneration of blood vessels does take place just prior to deep ulceration and is conspicuous during the period of deep ulceration.

Just as necrosis of epithelial cells (superficial ulceration) occurs following irradiation, so also could endothelial cells of blood vessels in the irradiated area undergo necrosis due to primary radiation damage. The considerable delay, before this is manifest, might be accounted for by a difference in the metabolism of the endothelial cells, possibly associated with a different rate of mitosis or the lesser exposure to irradiation of the deeper tissues by the technique used. Nevertheless, though hair loss is delayed by cortisone treatment, epidermal damage, producing superficial ulceration, does not appear to be affected. It is therefore difficult to accept that it is a primary endothelial lesion which is delayed and modified by cortisone treatment, when primary epithelial damage is virtually unaffected by this drug.

Degeneration of blood vessels may, however, be secondary to irradiation injury, possibly resulting from the effect on endothelial cells of toxic products of cells damaged or destroyed by the primary injury. It is conceivable that cortisone protects against such secondary damage to endothelial cells. There is evidence that cortisone protects blood vessels in other conditions. Ebert and Barclay (1953), observing the blood vessels in rabbit ear chambers, found that, during infection and serum sickness, cortisone gave better maintenance of vascular tone, reduced damage to vascular endothelium, and decreased emigration of leucocytes and exudate formation.

There is, however, much evidence against the view that " deep ulceration " is due to toxic products produced at the time of superficial ulceration. Firket (1951) produced localised CO_2 snow damage to guinea-pig skins. There was epidermal necrosis succeeded by gross hyperplasia closely resembling that described in the epidermis of irradiated guinea-pig skin (Mellett *et al.*, 1960). No second phase of necrosis, like that following irradiation, was observed. After a period of 2 months or more the epidermal hyperplasia gradually subsided. If, following CO_2 snow burns, a " diffusable toxin " was released from damaged epidermal cells, it would not appear, in these circumstances, to have caused subsequent damage to blood vessels or the hyperplastic epithelium. The fundamental difference in the nature of the trauma may alone account for the different result.

As further evidence against blood vessel damage being secondary to earlier

epidermal damage, it has been observed in a previous experiment (Houghton, Mellett, Jones and Walter, 1957) that if the area of skin to be irradiated is shaved with an electric razor just before irradiation, the ensuing superficial ulceration is more marked and occurs earlier ; the later deep ulceration is, however, quite unaffected in timing or degree (Houghton *et al.*, 1957). It may be inferred that the blood vessel changes and deep necrosis are not initiated or affected by the previous epithelial damage.

It may be the non-endothelial cell elements of small blood vessels which are principally damaged following irradiation. It has been shown by Wyman, Fulton and Shulman (1953), from direct observation of the hamster cheek pouch, that corticosteroids may influence the state of the blood vessels through their important effects on the connective tissue with which all vessels, including capillaries, are surmounted. Although fibrosis is unimpressive in the dermis generally before deep ulceration, there is some increase of fibroblasts and fibrous tissue and it may be that concurrent changes in the connective tissue coats of small blood vessels are sufficient to weaken their structure and impede function.

Zweifach, Shorr and Black (1953), observed the muscular venules of rats late in the syndrome ensuing upon adrenalectomy. These venules showed a pronounced tendency to rupture, presumably because of a deficiency in the perivascular connective tissue coat associated with corticosteroid deficiency.

Similarly Wyman *et al.* (1953) studied the behaviour of venules in the everted cheek pouch of a hamster following adrenalectomy. They found that the venules showed greater susceptibility to petechial formation in response to negative external pressure. Cortisone acetate (5–10 mg.) in intact animals gave rise to arterial vasoconstriction. The sensitivity of vascular smooth muscle (as indicated by response to nor-adrenaline and stimulation with micro-electrodes) was decreased and resistance of blood vessels to petechial formation with negative pressure was increased.

In the course of observations on the irradiated skin of guinea-pigs (Mellett *et al.*, 1960), a brown discolouration below the epidermal surface (presumably due to extravasated blood) was regularly noticed at the commencement of deep necrosis. The positive action of cortisone in protecting the integrity and function of small blood vessels described above may possibly provide a rationale for its apparent action in protecting the small blood vessels of the guinea-pig skin damaged by primary or secondary effects of X-irradiation.

While there is strong evidence that deep necrosis is due to degeneration of blood vessels, there can as yet be no firm conclusion as to the cause of this degeneration. It would seem possible that it is related to a change in the perivascular fibrous tissue.

Ross (1956), discussing radiation fibrosis of the lungs, points out that, while the skin reaction (superficial ulceration) is at its height one week after a four to five week treatment and has cleared to the maximal degree in three months, the changes seen radiologically in the lung fields occur 2–6 months later and may be unfolded over $1\frac{1}{2}$–2 yr. This may correspond to the second phase of deep ulceration associated with vascular changes seen in guinea-pig skin. This seems the more likely since the lung is composed largely of vascular tissue.

It is evident that a series of pathological changes initiated by irradiation continues for a considerable time after irradiation of any tissue. In an investigation of the value of cortisone as an adjunct to radiotherapy it would seem

appropriate to carry on treatment with cortisone long after the completion of radiotherapy.

SUMMARY

A total of 27 adult albino guinea-pigs were exposed to an X-ray dose of approximately 5000 r over a circular field 2·6 cm. in diameter on both flanks. Sixteen of these animals were given daily injections of 10 mg. cortisone acetate for 42 days commencing in the evening following irradiation. The remaining control group of 11 animals received no further treatment. On the 29th, 36th and 42nd days the irradiated areas of one side of 1 or 2 control animals and 4 cortisone-treated animals were excised completely. The remaining irradiated areas were untouched and their appearances were observed.

Hair loss was delayed in cortisone-treated animals. Desquamation occurred in all animals between the 20th and 40th days after irradiation ; some animals of both groups showed wet superficial (epidermal) ulcers at this stage. Deep ulceration of all layers of the skin was shown by 10 of the 11 control animals by the 49th day. Deep ulceration was markedly delayed in cortisone-treated animals ; 11 of the 16 showed no deep ulceration before the 64th day. In the interval between superficial and deep ulceration, 14 cortisone-treated animals showed rampart rings (due to hyperplastic hair follicle cysts) for long periods. This phenomenon was shown for a short period by 6 control animals.

For a given naked eye appearance the microscopic changes did not differ between cortisone-treated and control groups. Hyperplasia of the epidermis between superficial and deep ulceration was not diminished in cortisone-treated animals. The hyperplastic epidermis was maintained throughout the extended stage between superficial and deep ulceration and, macroscopically, rampart formation was therefore more prominent in this group. Deep ulceration, when it occurred in control or, after cessation of treatment, in cortisone-treated animals, was preceded and accompanied by degenerative changes in arterioles. While abnormal fibroblasts were seen in the dermis of control and cortisone-treated animals, there was no striking excess of fibrosis.

It is suggested that deep ulceration is caused by blood vessel degeneration. The means whereby blood-vessel damage may be caused following X-irradiation and the possible mechanisms whereby cortisone may prevent this damage are discussed. The hypothesis that vascular connective tissue is damaged by direct radiation injury and that the declaration of such damage is prevented by cortisone is favoured but not conclusively proved.

It is pointed out that cortisone acts on the long-lasting process of post-irradiation change in tissues and it is suggested that, where cortisone is used as an adjunct to radiotherapy for the prevention of damage to normal tissue, it must be given in adequate doses for very long periods.

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THE GREATER OMENTUM AS A SITE OF ANTIBODY SYNTHESIS

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THE greater omentum is a structure which has long excited the interest of surgeons and anatomists, and many theories have been advanced about its function. From clinical and post-mortem evidence, it is clear that the omentum plays an important role in surrounding and localising intraperitoneal foci of infection. The suggestion has also been made by Portis (1919) and Roberts (1955) that the omentum is an important site of antibody formation when the route of administration of antigen is intraperitoneal.

Walker, Thomson and Gray (1960) have reviewed the clinical and experimental work on this subject, and have described a series of studies designed to investigate the immunological potential of the omentum. Using rabbits immunized against *Salmonella typhi* by a course of intra-peritoneal injections, they showed that the titre of specific antibody in the omentum was higher than the serum titre, and higher than the titres of spleen, liver and peritoneum. In an animal from which the greater omentum had been removed surgically before immunization, the serum antibody level was considerably lower than that seen in intact animals. Portions of omentum from immunized animals were taken for culture *in vitro*, and the medium was found to contain measurable titres of specific antibody. Similarly, homotransplants of portions of omentum to non-immune recipients resulted in a serum antibody response which differed in pattern from that seen after the transfer of immune serum, or from the normal course of a primary immune response. Using the fluorescent antibody technique of Coons, Leduc and Connolly (1953), antibody-containing cells were demonstrated amongst the many lymphocytes and plasma cells which appeared in the omentum during immunization.

These findings provide powerful support for the suggestion that the greater omentum is an active site of antibody synthesis following intraperitoneal immunization. The experiments to be described here were designed to confirm these observations, and to provide direct evidence that the high antibody titres associated with the greater omentum are due, at least in part, to antibody synthesis by that tissue. To do this, it is necessary to show that the serum antibody titres seen in non-immune animals receiving grafts of immune omentum are not the result of the transfer of preformed extracellular antibody trapped in the tissue spaces of the graft, nor due to the release of intracellular antibody also present at the time of transfer. Preformed antibody might have originated in some other tissue, and its presence in the omentum could be the result of the inflammation caused by the intra-peritoneal injections of antigen.

Keston and Katchen (1956) and Humphrey and Sulitzeanu (1957) have used radio-isotope techniques with this same objective in view in studies of antibody

synthesis by tissue explants cultured *in vitro*, while Stavitsky (1956) employed similar methods in demonstrating antibody synthesis by lymph node cells transplanted into recipient animals. Humphrey and Sulitzeanu (1957) examined the time relations of antibody synthesis in various tissues of the rabbit. In order to measure the amount of preformed extracellular antibody carried over into the culture medium with the explant, the rabbits were injected with serum containing high titres of the specific antibody. This serum had been labelled with ^{131}I, and was injected 2–3 days before the explants were removed, to allow time for the labelled antibody to come into equilibrium with the extracellular antibody already present in the animal. The radioactivity of the antibody precipitated from the culture medium gave a direct measure of the extent to which preformed extracellular material had contributed to the precipitate. Active antibody synthesis *in vitro* was demonstrated by adding ^{14}C-labelled amino-acids to the culture medium, and estimating the radioactivity of the antibody subsequently isolated.

The present experiments followed this general design, with homografts of immune omentum to non-immune recipients replacing the explants into tissue culture. Recovery of specific antibody from the serum of the recipient was necessary in order to make the estimates of radioactivity, so a system giving precipitation of antibody with antigen was used. Pneumococcal capsular polysaccharide was chosen as the antigen, and the opportunity taken to repeat some of the experiments of Walker *et al.* (1960), which employed *Salm. typhi.*

The counting equipment available to us at the time of the experiment was not sensitive enough to give valid results with the low activities obtained. Autoradiographic emulsions have a high efficiency for low energy B-emitting isotopes such as ^{14}C, and long exposure periods make it possible to record very low activities at a level which is statistically useful. Autoradiography was therefore used to determine the levels of radioactivity found in the antigen-antibody precipitates.

MATERIALS AND METHODS

Adult white and sandy-lop rabbits were used. These were bred at the National Institute for Medical Research, Mill Hill, and at the Department of Experimental Pathology, Birmingham University, and were maintained on pelleted diet No. 18 (Bruce and Parkes, 1940) supplemented with hay.

Throughout the transplantation experiments, sandy-lop animals were used as donors, and whites as recipients. The serum antibody titres in normal, immunized animals were studied in 3 sandy-lop rabbits, and the serum titres in animals from which the greater omentum was excised surgically before the course of immunizing injections in a further 2 sandy-lops. Two donor rabbits and 2 recipients were used in the radio-isotope studies.

Formalin-killed type III pneumococci were obtained from the Standards Laboratory, Colindale, for the immunizing injections, and for the preparation of purified capsular polysaccharide by acid hydrolysis. The immunization consisted of six intra-peritoneal injections given at 3 day intervals. Each injection comprised 1 ml. of a suspension of pneumococci standardised to give an optical density equivalent to that of a No. 7 Brown's opacity tube.

Serum antibody was precipitated by the stepwise addition of small amounts of purified antigen until no further precipitation occurred. With this particular system, precipitation is not inhibited by a two- to three-fold excess of antigen, so that this procedure is unlikely to result in loss of antibody. After the final addition of antigen, the samples were left overnight at 20° for the deposition of antibody to continue to completion.

Procedure for radio-isotope experiments

A control, non-radioactive, antigen-antibody system was used to measure the level of radioactivity adsorbed upon a precipitate formed in serum containing labelled material.

Purified ovalbumin was used as the precipitating antigen. Dried rabbit anti-ovalbumin was available in the Department of Experimental Pathology. Each recipient animal was given an intravenous injection of 500 mg. of this, dissolved in 5 ml. of distilled water.

Rabbit serum containing a high titre of anti-pneumococcal (type III) antibody was labelled with ^{131}I by the rapid mixing method of McFarlane (1956). Each donor animal received 55 μc. of this labelled serum intravenously.

An acid hydrolysate of Chlorella protein, uniformly labelled with ^{14}C, was obtained from the Radiochemical Centre, Amersham, and 100 μc. administered intraperitoneally to each recipient rabbit.

On the 16th day after the first of the series of immunizing injections, the two donor rabbits were given sodium iodide in their drinking water, at a concentration of 50 mg./l. This was continued up to the time of transplantation, to reduce to a minimum the thyroidal uptake of radio-iodide liberated by the breakdown of the ^{131}I-labelled serum. On the 18th day, each donor rabbit was given 55 μc. of ^{131}I-labelled serum, intravenously. On the 20th day, the rabbits were killed. The thorax was opened immediately, the right auricle removed, and a cannula placed through the interatrial septum into the left atrium. Two litres of normal saline were perfused into the left atrium, and thence throughout the body, using the heart as a manual pump. This cleared most of the blood from the tissues, minimising the chance of including antibody-producing cells from the blood in the transplanted omentum, and removing the intravascular component of any preformed extracellular antibody. The greater omentum was then excised and weighed, and 7 g. transferred immediately to the abdominal cavity of a non-immune recipient rabbit, as a free, intraperitoneal graft.

Several hours after the transplantation, each recipient animal received 500 mg. of anti-ovalbumin intravenously. Blood samples were taken from each recipient for the estimation of the level of radioactivity in the specific antibody 48 hr. after transplantation. Any activity found in this sample could only be attributed to the ^{131}I-labelled serum, and would indicate a transfer of extracellular antibody in the graft. Immediately after withdrawing this sample, the two rabbits were injected intraperitoneally with 100 μc. each of ^{14}C-labelled amino acids. Further blood samples were withdrawn for autoradiography 8 hr., 5 days, and 7 days after the injection of labelled amino acids.

Procedures for autoradiography

Two areas $\frac{3}{4}$ in. square were marked with a diamond pencil on each of a number of microscope slides, and labelled A and C. The slides were carefully cleaned, and then dipped in a dilute aqueous solution of gelatin (gelatin, 5 g., chrome alum, 0·5 g., water to 1 l.), and allowed to dry.

The serum from each blood sample was divided into two equal portions. Pneumococcal capsular polysaccharide was added to the first until precipitation was complete, and ovalbumin to the second. The resultant precipitates were carfeully washed, and redissolved in 0·5 ml. each of distilled water. 0·1 ml. of this solution from the pneumococcal system was placed on the square marked A on the appropriate slide, and spread to cover the entire square. A similar amount of the control solution containing anti-ovalbumin was placed in the square marked C. Both were evaporated to dryness at room temperature. Each slide therefore carried the specific, endogenous, and the control, exogenous, antigen-antibody precipitates, derived from the same volume of serum.

Autoradiographs of these slides were prepared using Kodak AR–10 stripping film, as described by Pelc (1956), and also by applying Ilford K2 nuclear research emulsion by the painting technique of Belanger (1950). The slides from the first blood sample were prepared within 2 days of taking the sample. Both the AR–10 and the K2 autoradiographs of this sample were exposed for 28 days.

The slides from the subsequent blood samples were left for about 10 weeks before autoradiographs were prepared, to allow any ^{131}I activity initially present to decay away to insignificant levels. The AR–10 autoradiographs from these samples were exposed for 14 days, the K2 autoradiographs for 18 days. After development and fixation, they were mounted under coverslips for microscopy.

Grain counts were carried out on a photometric device to be described fully elsewhere (Rogers, unpublished). This employs a photomultiplier tube mounted on a microscope, which is fitted for incident illumination. The light output from each field viewed in this way is made up of two components : light reflected and scattered by the optical system, which

is constant for different fields on the same slide, and light reflected by the grains of metallic silver in the photographic emulsion. Above a threshold value, which represents the first component, the response of the machine increases linearly with increasing number of silver grains. Any difference in the observed light output between fields in the A and C squares of the same slide is due entirely to a difference in grain density. The readings are in the form of a galvanometer deflection, and are expressed in arbitrary units. To avoid bias in the selection of fields for measurement, the readings in each square were taken in 5 or 6 rows of 6 readings each. Two millimetres separated each reading from the next, and each row from the next. These readings have proved more accurately reproducible, and far quicker to take, than visual grain counts. The readings taken from one of the slides were checked against a series of visual grain counts.

The AR–10 stripping film is uniform in thickness over the entire slide, and it was expected that this would make it more suitable for quantitative work than the K2 emulsion. In fact, the photometric readings were much more variable, and, in general, higher with the stripping film. This confirmed the impression obtained visually that the grain densities were much more variable than with the painting technique. This high " background " of developed silver grains was presumably due to the greater degree of manipulation required in stripping film, and was sufficient to make accurate observations difficult with some slides. Table I

TABLE I.—*Photometric Readings Taken from K2 and AR–10 Autoradiographs of Precipitates from the Same Blood Sample, and Visual Grain Counts from a Different Series of Fields on the Same Ar–10 Slide*

Slide		Estimations of relative grain density				
		Mean	Number of observations	Standard deviation	Standard error	*P**
K2 slide	Square A	7·23	36	3·73	0·621	
	Square C	5·43	36	1·48	0·246	0·01–0·001
AR–10 slide—	Square A	6·43	30	6·03	1·100	
Photometric readings	Sauare C	3·04	30	2·80	0·510	0·01–0·001
AR–10 slide—	Square A	145·0	18	73·59	17·34	
Visual grain counts	Square C	102·0	18	53·17	12·53	0·50–0·02

* The statistic *P* was derived by Student's *t*-test.

compares the photometric measurements from a K2 slide, similar measurements from an AR–10 slide, and a series of visual grain counts from the latter slide. Both slides were of the same antigen-antibody precipitate, that from the blood sample taken 8 hr. after the administration of ^{14}C-labelled amino acids to one of the recipient rabbits. It will be seen that the photometric readings from the AR–10 slide had a higher standard deviation than those from the K2 slide. Both the series of photometric readings agreed well in showing a significantly higher mean reading in the A square than in the C square. This close agreement between the results with the two different techniques suggested that no gross error was introduced by the inevitable slight variations in thickness in the K2 emulsion. The visual grain counts, which were all carried out by the same observer, were from larger fields than the photometric readings. Approximately the same total area of emulsion was scanned by the two methods. The visual counts confirmed that the difference in photometric readings between the A and C squares corresponded to a difference in grain density.

In view of the high and unpredictable level of " background " found with the AR–10 slides, the results will be based on the K2 series of autoradiographs.

RESULTS

Table II shows the serum antibody titres of 3 normal rabbits during the course of immunizing injections, and the serum titres of 2 rabbits from which the greater omentum had been excised prior to the immunizing injections. It will be seen that the animals which lacked the omentum responded with lower serum titres to the intraperitoneal injection of antigen.

TABLE II.—*Serum Antibody Titres of 5 Rabbits Given a Course of Intraperitoneal Injections of Type III Pneumococci*

Rabbit	Days after first immunizing injection			
	5	10	15	20
1. Intact . . .	1 : 200	1 : 400	1 : 800	1 : 3200
2. „ . . .	1 : 50	1 : 100	1 : 800	1 : 1600
3. „ . . .	1 : 100	1 : 400	1 : 3200	1 : 6400
4. Without omentum .	1 : 50	1 : 50	1 : 100	1 : 800
5. „ „ .	1 : 50	1 : 100	1 : 400	1 : 400

Fig. 1 shows the serum antibody titres found in a non-immune recipient rabbit following transplantation of omentum from an immune donor. A measurable serum titre appeared within 6 hr., and this rose to a high level until between the 9th and 11th days when a sharp drop occurred. It seems reasonable to assume that destruction of the grafted omentum by the immune reaction of the host was responsible for this fall in antibody titre.

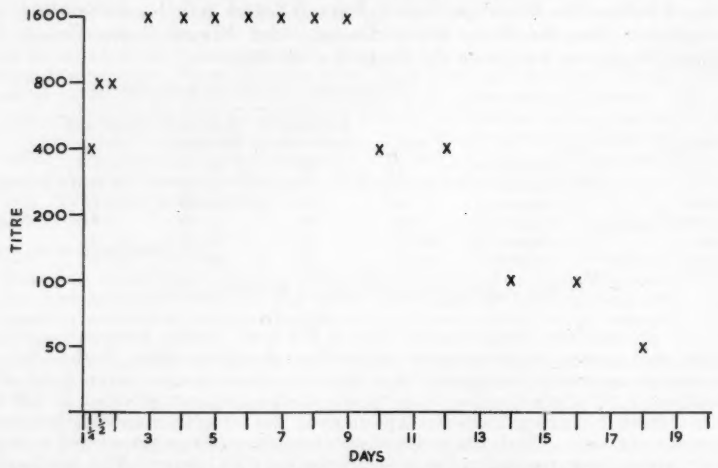

FIG. 1.—The serum titres of specific antibody in a non-immune rabbit that received an intraperitoneal graft of omentum from an immunized donor on day 1.

Table III presents the data obtained from the K2 autoradiographs derived from the blood samples of the first recipient animal. The means of the photometric readings in this series are shown graphically in Fig. 2.

Taking the results from the control, ovalbumin, system first, it will be seen that the readings from the samples obtained before the administration of ^{14}C-labelled amino-acids, and those 5 and 7 days later, were uniformly low. Student's *t*-test fails to show any significant difference between these figures. By contrast, the sample taken 8 hr. after the administration of ^{14}C gave a higher reading. Evidently, the precipitate which formed in the serum at this time carried down with it adsorbed radio-active material which was not removed by subsequent washing. Many of the labelled compounds present in the serum at this time were

TABLE III.—*Photometric Readings from Autoradiographs of Serum Samples from a Non-immune Rabbit Grafted with Immune Donor Omentum*

Slide		Estimations of relative grain density: Mean	Number of observations	Standard deviation	Standard Error	P
First sample—	Square A*	1·19	36	0·409	0·068	C > A
before injection of ^{14}C	Square C	1·66	36	0·684	0·114	<0·001
Second sample—	Square A	7·23	36	3·73	0·621	A > C
8 hr. after injection of ^{14}C	Square C	5·43	36	1·48	0·246	<0·01, >0·001
Third sample—	Square A	4·41	36	3·22	0·537	A > C
5 days after injection	Square C	1·85	36	1·29	0·215	<0·001
Fourth sample—	Square A	2·19	30	0·837	0·153	A > C
7 days after injection	Square C	1·91	30	0·496	0·091	0·2–0·1

* The specific antibody was in Square A, the control antibody in Square C.

presumably either catabolised and excreted, or incorporated into intracellular material by the time the 5 day sample was taken.

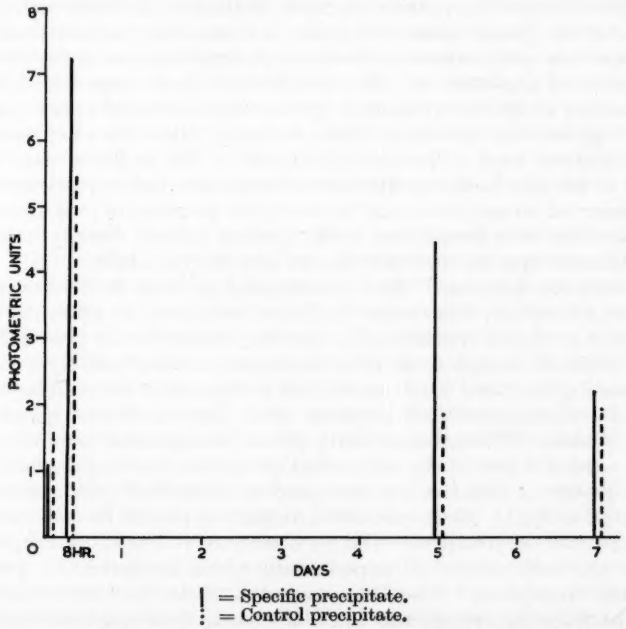

FIG. 2.—The means of the photometric readings from the K2 series of autoradiographs prepared from the serum samples of the first recipient animal. The first sample was taken after transplantation of omental tissue, and immediately before the injection of ^{14}C-amino acids.

Turning next to the readings obtained from the specific (pneumococcal) system, those from the first sample were lower than those for the control system. There was therefore no radioactivity in this precipitate which could be attributed to the ^{131}I-labelled serum given to the donor animal. Eight hours after the ad-

ministration of ^{14}C amino acids, the readings over the specific precipitate were higher than those over the control. This difference is statistically significant by Student's *t*-test ($P < 0{\cdot}01$, $> 0{\cdot}001$). In the autoradiographs of the 5 day sample, this difference was even more marked ($P < 0{\cdot}001$). By 7 days, readings over both precipitates were again equal.

The results from the second recipient rabbit were not quite so satisfactory, but they confirmed the general pattern seen in the first animal. Before the administration of ^{14}C amino acids, there was no significant difference between specific and control systems ($P < 0{\cdot}9$, $> 0{\cdot}8$). Eight hours later, the readings from the autoradiograph of the specific precipitate were higher ($P < 0{\cdot}1$, $> 0{\cdot}05$). By 5 days, this difference had become statistically significant ($P < 0{\cdot}01$, $> 0{\cdot}001$). At 7 days there was again virtually no difference between the two ($P < 0{\cdot}2$, $> 0{\cdot}1$).

DISCUSSION

The results presented here, utilising pneumococcal capsular polysaccharide as antigen, confirm the general pattern found by Walker *et al.* (1960) with *Salm. typhi*. The removal of the greater omentum before a course of intraperitoneal injections of antigen results in lower serum antibody levels than are seen in the intact animal. Transplantation of a portion of omentum from a donor immunized in this way to a non-immune recipient is followed by the appearance of specific antibody in moderately high titre in the serum of the recipient. But this observation may be interpreted in three ways. The antibody found in the recipient may result from the transfer of antigen in the graft, with subsequent antibody production by the host. The observed serum titres may be due to the presence of preformed antibody, either extracellular or intracellular, in the grafted tissue. Finally, the graft may actively synthesize specific antibody in the non-immune host.

Several workers have used this experimental pattern of the transfer of cells from an immune donor into a non-immune recipient, in order to investigate possible sites of antibody synthesis. Harris, Harris and Farber (1954), for example transferred popliteal lymph node cells from rabbits that had been inoculated in the corresponding foot, and found an antibody response in the recipient. Stavitsky (1956) found a similar antibody response after the transfer of lymph node and spleen cells. Oakley, Warrack and Batty (1954) transplanted a number of tissues, and made a careful study of the time relations of the resulting antibody response. In all three groups of experiments, the pattern of antibody formation resembled that illustrated in Fig. 1. Demonstrable antibody appeared far sooner than would be expected in a primary response. Oakley *et al.* were able to show a typical primary response by the host animal in experiments which favoured the persistence of antigen in the transplant. When this was seen, it did not occur until about 10 days after the transplantation, and could be clearly distinguished from the earlier antibody response attributed to the graft itself. In the present experiments, no such diphasic response was seen. Presumably the high antibody titres in the donor at the time of transplantation made it unlikely that antigen would persist in significant amounts. It seems very improbable then, that antibody formation by the host animal could account for the pattern of response found in these experiments.

Both Oakley *et al.* (1954) and Walker *et al.* (1960) studied the serum antibody titres in the non-immune recipient after the injection of serum from an immune

donor. In this type of experiment, where preformed extracellular antibody is deliberately transferred in considerable amounts, an initial antibody peak is found in the serum of the recipient, followed by a rapid exponential drop during the subsequent few days. The persistence of a high antibody titre in the recipient for 10 days, as in the experiments reported here, cannot be explained as the transfer of preformed extracellular antibody. This view is consistent with the failure to detect any radioactivity in the first serum samples from the recipient animals.

The possibility that the antibody titres seen in the recipients were due to the slow release of preformed, intracellular, antibody by the graft is also remote. Walker *et al.* heated portions of omentum, before placing them in the recipient, to a temperature sufficient to kill the cells, but not high enough to denature any preformed antibody present. The serum titres in the recipient showed a low peak 1–2 days later, falling away to zero in 5–6 days. This pattern, too, fails to correspond to that seen following grafts of living omentum. It may be argued that, in the latter case, when cells were transferred live, the process of lysis and liberation of intracellular antibody might have followed a different time course. In such a graft, however, many of the cells presumably would remain alive until about the 10th day, when widespread cell destruction would accompany the reaction of the host against the homograft. If the antibody circulating in the recipient had been preformed in the donor, and intracellular in location, a rise in serum titre might have been expected at this time of increased cell death. But the observed titres started to fall at this time, suggesting that the presence of live cells from the donor was necessary to maintain the antibody titres seen in the recipient.

It therefore seems highly unlikely that preformed antibody either extracellular or intracellular, contributed significantly to the serum titres of the recipients. On the other hand, the incorporation of ^{14}C amino acids into the antigen-antibody precipitates showed conclusively that molecules of specific antibody had been synthesised in the recipient animals. Stavitsky (1958) in a similar type of experiment, was able to demonstrate the incorporation of ^{35}S methionine into specific antibody in the non-immune recipient. It is interesting to note that, in the experiments reported here, there was no detectable radioactivity in the antigen-antibody precipitates 7 days after the injection of ^{14}C amino acids, although the serum titre of antibody remained high until after the 10th day. Humphrey and McFarlane (1954) have shown that isotopically labelled antibody molecules have a biological half-life in the rabbit of about 120 hr. In the light of this, it seems reasonable to interpret the results as demonstrating the loss by catabolism and excretion of the molecules of radioactive antibody over the course of 7 days, and their replacement by a population of antibody molecules synthesized after labelled amino-acids ceased to be available. It is difficult to avoid the conclusion that the greater omentum is the site of active antibody formation when the antigen is administered into the peritoneal cavity.

The sites of antibody synthesis fall into two main groups. The first comprises the regional lymph nodes, spleen, lungs, and bone marrow, in which active synthesis may take place in response to an antigen injected elsewhere in the body. Humphrey and Sulitzeanu (1957) and Askonas and Humphrey (1958) have shown that, in the rabbit, an intravenous injection of antigen is followed by antibody production in the spleen, lungs and bone marrow, in descending order of magni-

tude. The subcutaneous injection of antigen results in antibody synthesis in the regional lymph node (Harris *et al.*, 1954 ; Coons *et al.*, 1953 ; Oakley *et al.*, 1949). The second group consists of certain tissues which respond to the local injection of antigen by the local formation of antibody. Askonas and Humphrey, for example, were able to show that the granuloma which develops at the site of intramuscular injection of antigen mixed with Freund's adjuvant is the major source of antibody in this instance. Oakley, Batty and Warrack (1951) and Oakley *et al.* (1954) demonstrated local antibody synthesis in fat from several parts of the body, including the omentum, skin, and skeletal muscle. These three tissues appeared to retain antigen longer than the other tissues studied at the same time, as judged by their ability to cause a primary response on transplantation into a non-immune host. It is interesting to note that adipose tissue appeared to be incapable of antibody synthesis until 24 hr. after the injection of antigen. Oakley *et al.* related this change in immunological ability to the infiltration of cells around the injection site that accompanied early stages in the formation of the granuloma.

The greater omentum resembles both groups of antibody-forming tissues. It can respond to the injection of antigen directly into its substance by the local synthesis of antibody. It also synthesizes antibody when the antigen is administered into the peritoneal cavity.

SUMMARY

Serum antibody titres were studied in rabbits given a series of intraperitoneal injections of type III pneumococci. The titres were lower in animals from which the greater omentum had been removed than in intact animals.

Portions of the greater omentum were transplanted from rabbits immunized by the intraperitoneal route into the abdominal cavities of non-immune recipients. A characteristic rise and fall in serum antibody titre followed, suggestive of active antibody synthesis by the graft within the host animal.

Rabbit serum containing the specific anti-pneumococcal antibody was labelled with ^{131}I, and administered to immune donor animals before transplantation of portions of omentum to non-immune recipients. Autoradiographic examination of the serum antibody found later in the recipients failed to detect any radioactivity.

^{14}C-labelled amino-acids were administered to non-immune rabbits that had received intraperitoneal grafts of omentum from immunized donors. Autoradiographic examination of the specific antibody found in the serum of the recipients showed that it contained significant levels of radioactivity 8 hr. and 5 days later.

These findings are discussed, and it is concluded that the greater omentum is an important site of active antibody synthesis following the intraperitoneal injection of antigen.

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A MAINTENANCE MEDIUM FOR TISSUE CULTURE VIRUS STUDIES

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THE use of tissue culture techniques for studying viruses has led to a special requirement for a type of medium usually referred to as maintenance medium. Its purpose is to keep the virus-infected cells healthy and susceptible to the virus, sometimes for long periods, without itself having any inhibitory effect on the virus. These conditions are not necessarily, or indeed generally, satisfied by the standard media used for growing the cultures.

The liver digest medium to be described has given good results as a maintenance medium with a number of different cells and viruses.

DESCRIPTION OF MEDIUM

Preparation of stock liver digest

The method of preparation was originally (Smith, 1954) adapted from the instructions for peptic digests given by Parker (1938). It has been modified at various times, in accordance with the quantities required and the facilities available, without any significant variations in the quality of the product. The method outlined is used in these laboratories for large scale production, but would be equally suitable for small amounts.

Fresh horse liver is minced and suspended by means of a blender in 0·05 N HCl (250 g. of liver to 1 l.) and pepsin powder (250 mg. to 1 l.) is added. The proportions do not appear to be critical. The mixture is then incubated for 24 hr. Stirring or shaking during the first few hours is advantageous, but agitation during the whole period is not necessary. Although the low pH discourages most contaminants it is usual to add some volatile preservative such as chloroform (2·5 ml. per l.).

The use of horse liver was determined largely by its availability. Early small scale batches were made from rabbit liver and these gave good results. There seems no reason to doubt that any convenient source of liver could be used. The freshness of the liver is important and it is best used immediately on removal.

After digestion the mixture is boiled for a few minutes to destroy enzymes, precipitate protein and remove the preservative. The pH is brought up to about 7·0 by the addition of NaOH and the digest is freed from solid residue by filtration through cloth. Centrifugation at about 2000 r.p.m. has been found convenient for small volumes. If this crude digest is to be stored it is sterilized by Seitz filtration.

For use the digest must be ultrafiltered. A collodion membrane for this purpose is made as follows. A dry Berkefeld type filter candle of a suitable size

is immersed for four minutes in an 8 per cent solution of collodion in glacial acetic acid. It is then withdrawn, allowed to drain and immersed in water, when a membrane is formed on the surface of the candle. The membrane and candle are washed free of acetic acid by drawing water through them.

Ultrafiltration, by drawing the digest in through the candle by suction, is done in a refrigerator as complete sterility is difficult to achieve. About 90 per cent of the starting volume can usually be drawn through before concentration of the residue clogs the membrane. After 48 hr. the growth of contaminants often becomes troublesome and the size of the candle, or candles, is chosen to enable the process to be completed within this period. A " Cellophane " membrane can be used but is very much slower.

The ultrafiltrate must be adjusted empirically to the tonicity of culture media. A simple method which has been used in the absence of apparatus for freezing point determination is to make a series of parallel dilutions of digest and of normal saline and to determine the dilution at which each haemolyses a sample of red cells. The necessary adjustment to the digest to equate it with the saline can then be estimated with sufficient accuracy. Normally a slight dilution is needed but, if necessary, the digest may be concentrated by boiling.

The pH is finally brought to 7·0 and the product sterilized by Seitz filtration.

The crude digest may be made in large quantities, stored at 4° for several months and ultrafiltered in successive lots as required. The final product is given a shelf life of about 1 month at 4°, but there is some evidence that this may be a conservative estimate of its stability.

Composition

Analyses of a number of successive batches of the ultrafiltrate gave total nitrogen figures ranging from 2·65 to 3·31 g. per l. and amino nitrogen to total nitrogen ratios from 51·4 to 56·0 per cent. Although no effort is made to control the course of the combined peptic digestion and autolysis the results are remarkably constant. A digest obtained from liver would be expected to contain a large number of active ingredients not included in this simple nitrogen analysis. These have not been investigated, but the uniform behaviour of batches in use suggests that the tendency to a constant end point affects these components also.

An analysis by paper chromatography of the amino acids in comparison with those of lactalbumin hydrolysate, commonly used in growth media (Melnick and Riordan, 1952), showed a striking similarity in pattern between the two substances, the only marked difference being an excess of glycine in the liver digest. The total amino acid level in the complete maintenance medium is about half that in the usual 0·5 per cent working solution of lactalbumin hydrolysate.

The crude digest, before ultrafiltration, contains glycogen and about 0·1 to 0·5 g. per l. of protein among its large molecule components. As it is much less satisfactory than the ultrafiltrate, especially in medium with a low serum content, it is probable that toxic substances, perhaps bound to the large molecules, are removed by the ultrafiltration.

Complete maintenance medium

The liver digest is used at a 10 per cent dilution in Earle's balanced salt solution to form the basic maintenance medium. In some systems a level of 5 per cent has proved adequate.

RESULTS

Over a period of about 5 years the liver digest medium has been the principal maintenance medium used in these laboratories for primary monkey kidney cells (also Clarke, Goffe, Stuart-Harris and Herzog, 1958). For titrations of poliovirus the cultures are kept on a serum free medium for periods of up to 12 days from infection. For safety testing of poliomyelitis vaccine, cultures are maintained for periods of up to 28 days with weekly medium changes, again on serum free medium. In our hands the liver digest medium has given better maintenance of these cells than either a lactalbumin hydrolysate medium (on which, with added serum, the cells are usually grown) or S.M.199 (Morgan, Morton and Parker, 1950). The medium has been equally successful with monkey kidney cells used for growth and assay of a number of viruses, such as B virus and other simian viruses.

For plaque assays on monkey kidney cells an agar overlay containing 5 per cent of the liver digest and 0·2 per cent of bovine albumin Fraction V has been found satisfactory (Goffe, personal communication).

Other primary cells which have been maintained on this medium are dog kidney, lamb kidney, rabbit kidney and pig kidney. The last have generally been found to require serum.

Cells of established lines such as H. Ep–2 (Moore, Sabachewsky and Toolen, 1955) are in general more difficult to maintain for any length of time. A liver digest medium with the balanced salt solution modified to contain 0·2 per cent of glucose and with added yeast extract has given fairly good results, even in the absence of serum. Such a medium with 5 per cent of liver digest and with the addition of 1 per cent of calf serum is used routinely (Laurence, personal communication) for work with measles virus in H. Ep–2 cells.

In the course of this work there has been no evidence of decreased sensitivity of cells to virus or of interference with virus in this as compared with other media. This has been confirmed by direct comparisons with S.M.199 in the case of poliovirus in monkey kidney cells and of measles virus in H. Ep–2 cells.

DISCUSSION

The component of a tissue culture medium most commonly prejudicial to virus work is the serum, and maintenance media often consist of the normal growing media with the serum omitted. Since serum is in most cases an essential medium constituent, its omission would be expected to curtail the healthy life of the cells. The usefulness of the liver digest is primarily due to its ability to keep cells healthy with little or no serum present.

It is sometimes possible to select a serum which has been screened beforehand and found free from activity against the virus concerned, or serum substitutes such as bovine albumin (Sabin, Hennessen and Winsser, 1954) or skim milk (Baron and Low, 1958) may be used. Such modifications of the normal media may succeed in reconciling the needs of cells and virus but they tend to retain conditions which encourage cell growth, and this leads to a problem in cell maintenance which is not often considered. The ideal culture for most virus work is a monolayer of cells, and once this is attained further cell growth is of no value and is usually undesirable. A multiple cell sheet is difficult to examine critically

and, particularly in the case of epithelial lines, cells which lose contact with the glass tend to round up and further to obscure the picture. In mixed primary cultures the three dimensional conditions favour the fibroblasts which, as they come to predominate, develop tensions which may cause large areas of the cell sheet to pull off the glass. Such conditions may render a culture of healthy cells unfit for virus work. The liver digest medium has the merit of promoting growth less than media which were selected initially for this very property. Also the impression has been gained that mixed cultures develop relatively fewer fibroblasts on the liver digest medium and so maintain a more stable cell sheet.

SUMMARY

A maintenance medium for tissue culture virus studies is described. It consists basically of a 5 to 10 per cent dilution of a liver digest ultrafiltrate in balanced salt solution, and a method for the preparation of the digest is given. The special properties of the medium are that it maintains cell structures in good health in the absence of serum and that at the same time it gives relatively little growth stimulation.

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THE PSEUDOINTIMA LINING FABRIC GRAFTS OF THE AORTA

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DISEASED segments of the aorta and other large blood vessels in man are now often replaced by tubes of cloth woven or knitted from various artificial fibres (Voorhees, Jaretzki and Blakemore, 1952; Kinmonth, Taylor and Lee, 1955, and many other workers). There is general agreement that grafts made of such material are preferable to those formed from natural blood vessels or from impermeable plastic tubes. In connection with these advances in vascular surgery, a number of investigations have been undertaken to study the results of implanting such grafts into experimental animals, chiefly dogs. From the earlier investigations (Harris, Shumacker, Siderys, Moore and Grice, 1955; Sauvage and Wesolowski, 1955; Shumway, Gliedman and Lewis, 1955; Creech, Deterling, Edwards, Julian, Linton and Shumacker, 1957; Harrison, 1957; Szilagyi, Whitcomb and Shonnard, 1957; Szilagyi, France, Smith and Whitcomb, 1958) it was established that these grafts eventually become lined by a layer of tissue which is usually referred to as the pseudointima and contains both cells and fibres, but it was not clear whether or not the pseudointima was lined by endothelium. In two recent studies, by Meijne (1959) and by Mackenzie and Loewenthal (1960), the tissue lining fabric grafts of the aorta has been examined *en face* after staining by silver nitrate. In both cases the investigators were able to describe and illustrate a layer of flattened cells closely resembling endothelium which appeared to have developed from the endothelium lining the aorta at the ends of the graft.

The present investigation was undertaken to supplement the previous work. In view of increasing interest in the surgical possibilities of replacement of diseased arteries by fabric grafts, it is desirable to ascertain as far as possible the nature of the tissue which ultimately lines them. Moreover, these grafts provide an experimental situation in which the capacity for regeneration of various structural elements of the aortic wall can be studied. The demonstration by Jordan, DeBakey and Halpert (1958) that lesions resembling atheroma can develop in such grafts in hypercholesterolaemic dogs provides an additional reason for supposing that it might be of value to know more about the structure of the pseudointima.

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The baboon was used in the present investigations because it is an animal of convenient size for aortic grafting and because it is known that it sometimes develops small arterial lesions reminiscent of the milder forms of atheroma in man (Lindsay and Chaikoff, 1957 ; Gillman and Gilbert, 1957 ; McGill, Strong, Holman and Werthessen, 1960).

METHODS

Animals.—Wild baboons (*Papio doguera*) were captured in Kenya in an area near the confluence of the Kibwezi and Athi rivers by Mr. H. R. Stanton. Fully grown animals were used, weighing between 30–46 kg. Their age could not be estimated with any useful degree of exactitude. Grafts were inserted in the lower abdominal aorta by S. J. G. at Mr. Stanton's camp near Kibwezi. The animals were later transported to the Urschel Memorial Laboratory, Southwest Foundation for Research and Education, San Antonio, Texas, U.S.A., where 8 were killed and examined by H. W. F. and J. C. F. P. approximately 10 weeks after operation. Specimens were taken to Oxford for histological examination.

Operation.—The animal was anaesthetized and a left paramedian incision was made in the lower abdominal wall. The intestines were packed upwards out of the pelvis and lower abdomen. The peritoneum was incised over the aorta from its bifurcation up to the origin of the inferior mesenteric artery. Potts-Smith patent ductus clamps or similar vascular forceps were applied to the aorta just below the inferior mesenteric artery and just above the bifurcation and the intervening part of the aorta was removed. The excised portion of the vessel measured 2–4 cm. A knitted polyethylene glycol terephthalate (" Dacron ") graft was selected which had a diameter slightly larger than that of the excised part of the aorta : either the 6 mm. or the 8 mm. size was used. (This material will be more familiar to English readers under the name " Terylene ". A short account of its discovery and manufacture was given by Goadsby in 1952.). The graft was soaked in some of the animal's own blood so that a clot was formed within the pores of the knitted fabric. (This step greatly reduces blood loss later on.) The length of the graft was such as to put it under mild tension. An end-to-end anastomosis was made, the proximal end being connected first, and 2 continuous over-and-over silk sutures were used at each suture line. The proximal clamp was removed momentarily to allow blood to fill the graft and to clot while the distal anastomosis was being carried out. Just before the last few stitches were inserted an attempt was made to remove all free clot from within the graft, and the graft was flushed by again removing the proximal clamp momentarily. The final stitches were then quickly put in and the clamps were removed. The peritoneum was stitched together loosely over the graft and the abdominal wound was closed. No dressings were applied.

Histological techniques.—(i) The perfusion method described by Poole, Sanders and Florey (1958) was used with the aorta *in situ* to stain the endothelial " cement lines " with silver nitrate and to fix the aortic tissues with formalin. The animal was restrained in a squeeze cage and given an intramuscular injection of the tranquillizing drug 1-(1-phenylcyclohexyl) piperidine monohydrobromide (" Sernyl ", Parke Davis ; see Catenacci, 1958 ; Chen, 1958). It was later transferred in a somnolent condition to an operating table and anaesthetized with pentobarbitone sodium solution given both intramuscularly and intravenously. It was then given 5000 units of heparin by intravenous injection. A carotid artery and a femoral artery were cannulated with polyethylene tubing (size 4 polythene cannula manufactured by Allen and Hanbury, Ltd.) and the animal was then killed by injecting a solution of pentobarbitone sodium into the cephalic vein. By means of syringes attached to the carotid cannula the aorta was washed through successively with 500 ml. of 5 per cent glucose, 100 ml. of 0·25 per cent silver nitrate, 100 ml. of 5 per cent glucose, 500 ml. of 3 per cent cobalt bromide containing 1 per cent ammonium bromide, and finally with 10 per cent formalin, the formalin perfusion being continued from a reservoir for 1–2 hr. After fixation the aorta, including the graft, was tied across metal frames, removed from the body, slit open and pinned out flat on polyethylene sheeting. Its endothelial surface was then examined with a dissecting microscope.

(ii) Specimens prepared as in (i) were dehydrated in alcohol and cleared in clove oil to facilitate microscopical examination of the endothelial " cement line " pattern. Clove oil was better for clearing than cedar wood oil.

(iii) From specimens prepared as in (i) small areas of the pseudointima were dissected off the inner surface of the cloth tube, cleared in xylene and mounted flat in dammar. These preparations did not reveal any structures which were not visible in whole-thickness preparations cleared in clove oil. They could, however, be photographed more easily.

(iv) Pieces of pseudointima were prepared as in (iii) and embedded in ester wax (Chesterman and Leach, 1956). Sections were cut perpendicular to the endothelial surface in the long axis of the aorta and were further stained with haematoxylin and eosin, so that the endothelium, complete with silver lines, could be examined in section.

(v) Häutchen preparations of endothelium (*i.e.* layers of endothelium largely free of other tissue) were made both from normal aorta and from tissue lining the graft by the method described by Poole, Sanders and Florey (1958).

(vi) Two animals were killed rapidly by injecting pentobarbitone sodium into the cephalic vein. The segment of aorta containing the graft was removed as quickly as was consistent with gentle handling and portions were fixed in Caulfield's medium for electron microscopy (Caulfield, 1957) and in Helly's solution for the preparation of histological sections to be examined by light microscopy. The fixed material was transferred to 70 per cent alcohol and sent from San Antonio to Oxford to be embedded in methacrylate and in araldite, with and without prior treatment with phosphotungstic acid. "Gold" and "silver" sections were cut with glass knives on a Huxley microtome, floated on distilled water and mounted on either formvar-coated or plain copper grids. The material was observed and photographed in a Philips EM 100 B electron microscope using 60 kV.

The material fixed in Helly's solution was embedded in ester wax. Sections were cut perpendicular to the endothelial surface in the long axis of the aorta and stained with haematoxylin and eosin, haematoxylin and Van Gieson's stain, Heidenhain's iron haematoxylin, Mallory's phosphotungstic acid haematoxylin, Lendrum's acid picro-Mallory method, Weigert's long elastin stain, Verhoeff's elastin stain and Gordon and Sweet's reticulin stain.

RESULTS

General observations

Fig. 1 shows the appearance of the lower end of a baboon's aorta, complete with graft, after it had been opened and cleared. The graft was surrounded by connective tissue and lined by a pseudointima of variable thickness. The general disposition of tissue in relation to the grafted material is seen in Fig. 2 which shows part of a section cut perpendicular to the inner surface of a graft. Many foreign body giant cells were present in immediate relation to the graft and in some places polymorphonuclear leucocytes as well (Fig. 3).

Endothelium

The aortae of 6 animals were perfused with silver nitrate, so that after they had been opened the endothelial "cement line" pattern could be observed. (Reasons for supposing that the silver lines do not in fact represent a cement substance, as was previously supposed, have been given by Buck (1958) and by Florey, Poole and Meek (1959). Unfortunately no acceptable term is at present available as an alternative to "cement lines".) This pattern was complete over the whole inner surface of the graft. No discontinuity could be seen in the neighbourhood of the suture lines; the normal pattern over the real aorta merged imperceptibly with that over the graft (Fig. 4 and 5). The presence of a silver line pattern is not unequivocal evidence for the presence of endothelium. However, the examination of Häutchen preparations showed that the network of silver lines enclosed cells morphologically very similar to normal endothelial cells (cf. Fig. 6 and 7). The newly-formed endothelium in places showed large, irregular multinucleate cells (Fig. 8) reminiscent of those found in regenerating aortic

endothelium in the rabbit (Poole *et al.*, 1958, 1959). Similar cells were seen by Meijne (1959) in endothelium lining nylon grafts.

In histological sections of specimens in which the lining of the vessel had been stained by silver nitrate (Fig. 9) the characteristic appearance of endothelium is again demonstrated. Electron micrographs of the intimal surface show that it is lined by cells having the appearances associated with endothelium. Thus junctions between cells have the typical sinuous character and the plasma membranes of the adjacent cells are in close proximity to one another (Fig. 10). Some superficial cells contain many small vesicles such as are found in endothelium (Fig. 11). An incomplete basement membrane was seen.

Arterioles and capillaries arising from the lumen of the graft

An unexpected finding was that small branches, of arteriolar and capillary size, can be seen coming off the lumen of the graft (Fig. 12, 13 and 14). The internal diameters of these branches ranged from approximately 300 μ down to approximately 10 μ. They were particularly numerous near the suture lines but some could be seen in all regions of the graft. Most of them could be traced through interstices in the cloth but their peripheral connections could not be made out.

Cellular and fibrous components of the pseudointima other than endothelium

Many cells with elongated nuclei were seen lying beneath the endothelium (Fig. 9). These cells could not be identified by their appearance in histological sections examined by light microscopy. They are set in a matrix of fibrous material of which 2 components can be identified with reasonable certainty by light microscopy : elastin (Fig. 15) and collagen (Fig. 16). A large amount of collagen was present and so it is reasonable to suppose that some of the elongated cells were fibroblasts. Although elastic tissue is abundant it does not appear to be organized into fully developed elastic laminae. Some tendency towards a laminar arrangement can however be seen in places (*e.g.* Fig. 2). In sections stained with Gordon and Sweet's reticulin stain a few reticular fibres could be seen just beneath the endothelium but not elsewhere.

Further information about the nature of the cells and fibres was provided by electron microscopy. In a field at moderate magnification (Fig. 17) the majority of the sub-endothelial cells show the structure characteristic of smooth muscle. At a higher magnification both the myofibrils and the areas of darkly staining material usually found in smooth muscle cells can be seen (Fig. 18 and 19). Typical collagen fibres are seen in all the electron micrographs reproduced here. Elastic tissue is also seen in all these fields, especially those shown in Fig. 10, 19 and 20. In Fig. 21 some fibrillary material is seen just beneath the endothelium. It is not clear what it is, but it may correspond to the reticular fibres seen by light microscopy in the same position. Similar material is seen in part of the field shown in Fig. 10. Fine fibres were also seen in the deeper layers (Fig. 20). These may be of a different nature from the sub-endothelial fibres.

DISCUSSION

Growth of endothelium

It is known that capillary endothelium can grow quite rapidly : Clark (1931) estimated the speed at 0·1–0·6 mm. *per diem* in the transparent rabbit ear chamber.

In contrast, such evidence as is available about the growth of aortic endothelium suggests that its rate of growth is slow. Altschul (1954) concluded that it was doubtful if aortic endothelium could regenerate to any significant extent. However, Poole *et al.* (1959) were able to show that when endothelium was removed mechanically from the distal 2 cm. of the rabbit's aorta the endothelial lining slowly regenerated, but healing took about a year to complete—an overall speed of about 0·05 mm. *per diem*, or half this amount for each advancing edge. It was therefore surprising to find that in the baboon an aortic graft had apparently been lined with endothelium within 10 weeks. If the endothelium grows inwards from the edges of the graft, it can apparently do so at at least ten times the rate found in the rabbit. The reason for this difference is not clear. It would be strange if a piece of cloth provided a better matrix for endothelial growth than the tunica media, but it is conceivable that endothelium grows more rapidly over briskly regenerating tissue. The rate of endothelial growth in nylon grafts in various regions of the dog's aorta described by Meijne (1959) was apparently less rapid than in the present investigation but more rapid than in the rabbit experiments where the endothelial lining was removed from a segment of aorta. These apparent discrepancies may be due to species differences between the rabbit, the dog and the baboon. Possibly the endothelium lining fabric grafts does not grow exclusively from the aorta at the ends of the graft but arises in some other way. Until these possibilities have been explored it is unwise to draw any general conclusions about the speed of regeneration of aortic endothelium from the findings in any particular experimental situation.

While the present observations do not show the stages by which the graft comes to be lined by endothelium they provide grounds for supposing that the lining layer is composed of true endothelium in the sense that the cells are indistinguishable by any known criterion from authentic endothelial cells. In this respect most of the previous investigations have been inconclusive. No useful purpose would be served by reviewing the earlier studies in detail since they all suffer from the same technical limitations. These are that: (1) the material was fixed after it had been removed from the carcass; (2) morphological studies were confined to the examination by light microscopy of ordinary histological sections. The implications of these technical drawbacks are as follows.

(1) Endothelium is fragile. When a portion of a large vessel is removed by ordinary careful handling, the endothelium is often damaged and may appear deficient in places; sometimes sections cut from such material show only tattered fragments of endothelium instead of a continuous sheet. In the present investigation great care was taken in handling the specimens, the endothelium being fixed by perfusion and the aorta being steadied by tying across a metal frame before cutting it free in those specimens stained with silver. Even so it was found that normal endothelium was easily detached.

In the present investigation the cells lining the graft were seen in Häutchen preparations to look like normal endothelial cells, except that they were slightly less regular in shape and size and often contained more than one nucleus. These are the same differences from the normal that were previously found to be characteristic of regenerating aortic endothelium in the rabbit (Poole *et al.*, 1958, 1959). Thus the finding of these abnormalities reinforces the view that the lining layer of the graft is composed of endothelium.

(2) Because endothelial cells are long and broad but relatively very thin their

appearance in histological sections cut perpendicular to the inner surface of a large vessel is insufficient for determining their shape and size. Preparations where they can be seen *en face* are much better in this respect. Endothelial cells seen in section by light microscopy cannot be identified with certainty. In electron micrographs it is easier.

Most of these criticisms do not apply to the work of Meijne (1959) or of Mackenzie and Loewenthal (1960) who illustrated the appearances characteristic of endothelium seen *en face* after silver nitrate staining. The more detailed morphological studies reported here fully confirm their belief that true endothelium is present.

The structure of endothelial cells lining arteries has been investigated with the electron microscope by Moore and Ruska (1957), Buck (1958) and Florey (1960*a*, *b* and *c*). Their appearance in different arteries and in different animal species is similar. They are flat cells with nuclei which bulge into the lumen of the vessel; they are in close apposition, the junctions between them usually being sinuous, the mitochondria are small and not numerous; "pinocytic" vesicles are present but typical endoplasmic reticulum is inconspicuous or invisible; a basement membrane is present in some situations. No single feature is specific for endothelium but the appearance of the cells lining the graft is more like that of endothelium than of macrophages or fibroblasts.

Small branches arising from the lumen of the graft

A number of previous investigators have alleged that small vessels, down to capillary size, can in certain circumstances pass from the lumen of the aorta and other large arteries into the intima. Rokitansky (1852) stated that old atherosclerotic plaques became vascularized in this way. Woodruff (1926) noticed occasional small channels arising from the lumen in the ascending aorta of the dog, Robertson (1929) in the ascending aorta of man, and Winternitz, Thomas and Lecompte (1938) in the aorta of a cow and in the normal human aorta. Leary (1934) and Paterson (1936, 1938) described similar vessels in human coronary arteries. Geiringer (1951*b*) denied that they were found in healthy human vessels and thought that Winternitz *et al.*, might have created artifactual channels by their high-pressure injection technique. He reviewed his own earlier work (1949, 1951*a*) and concluded that when the intima thickened rapidly by mural thrombosis (the mechanism whereby Duguid, 1946, supposed that atheromatous plaques usually increase in size) then the thickened area was vascularized by branches from the lumen; when, on the other hand, the intima thickened slowly its deeper layers gained a new blood supply by extension of the vasa vasorum. Crawford and Levene (1952) studied the fate of mural thrombi in the human aorta and concluded that they were partly organized by capillaries entering them from the lumen. Levene (1956) found similar appearances in coronary arteries and illustrated capillaries and sinusoids in an apparently convincing manner.

Nevertheless many workers have considered that the existence of small vessels down to capillary size was improbable owing to their having to withstand arterial pressure. The present study shows clearly that very small vessels can leave the lumen of an aortic graft and any theoretical arguments against their existence are irrelevant. It follows that there is no theoretical reason to suppose that the appearances illustrated by Levene and certain previous workers are not what

EXPLANATION OF PLATES

FIG. 1.—The lower end of the abdominal aorta of a baboon with a graft in place. The vessel had been slit open and pinned out flat, dehydrated in alcohol and cleared in clove oil. ×2·2.

FIG. 2.—Section through the wall of an aortic graft. The fibres of the cloth appear black. The pseudointima is in the upper part of the field. In the lower part the graft is seen to be surrounded by loose connective tissue containing many blood-vessels. Weigert's long elastin stain. ×45.

FIG. 3.—Foreign-body giant cells and inflammatory cells near fibres of the fabric (lower left). Heidenhain's iron haematoxylin. ×360.

FIG. 4.—Endothelial "cement-line" pattern at the edge of the graft. The "cement-lines" were stained by silver nitrate, and the whole specimen has been cleared in clove oil. The dark bands are silk stitches. The vessel whose orifice is seen in the upper right part of the field corresponds to no normal aortic branch and is presumably newly formed. ×45.

FIG. 5.—From the same specimen as Fig. 4. This field was near the middle of the graft. The pattern of the knitted fabric can be seen slightly out of focus beneath the endothelium. ×35.

FIG. 6.—Häutchen preparation of endothelium from the normal baboon aorta. Silver and haematoxylin. ×360.

FIG. 7.—Häutchen preparation of endothelium lining the graft. Silver and haematoxylin. ×360.

FIG. 8.—Häutchen preparation of endothelium lining the graft. Several multinucleate endothelial cells are shown. In Fig. 7 and 8, the small densely-staining nuclei are those of blood monocytes. Silver and haematoxylin. ×360.

FIG. 9.—Section through the inner part of the pseudointima, cut perpendicular to the inner surface and in the long axis of the vessel. Endothelial lines had been stained by silver nitrate by perfusion prior to fixation. N: endothelial nuclei; S: silver lines. Haematoxylin and eosin. ×720.

FIG. 10.—Electron micrograph of a section through the inner part of the pseudointima. E: endothelial cell. CJ: junction between 2 adjacent endothelial cells. The endothelial cells contain many vesicles, have an incomplete basement membrane (BM) and rest on some fine fibrous material (FF) which has no characteristic structure. Elastic tissue (EL) and collagen fibres (COL) are seen deep to this fine fibrous layer. Tissue treated with phosphotungstic acid before embedding in Araldite. ×20,000.

FIG. 11.—Electron micrograph of a section through the inner part of the pseudointima. Part of an endothelial cell is seen. There are numerous vesicles (V), many of which are arranged along the inner plasma membrane. Other structures as in Fig. 10. ×28,000.

FIG. 12.—Small branches arising from the lumen of the graft. The inner part of the pseudointima has been dissected away from the cloth tube. ×145.

FIG. 13.—From a specimen similar to that illustrated in Fig. 12. The photographic plate was exposed at two different focal planes so that the silver line pattern of the branches is partly visible in the deeper focal plane. ×145.

FIG. 14.—Häutchen preparation of endothelium lining a graft including the proximal part of a branch. The Häutchen was mounted upside down so that the endothelium is viewed from its deep surface. The branch has been torn away from its deep connection. Silver and haematoxylin. ×320.

FIG. 15.—Elastic tissue in the pseudointima. Weigert's long elastin stain. ×1500.

FIG. 16.—Bundles of collagen fibres in the pseudointima. Van Gieson's stain. ×1500.

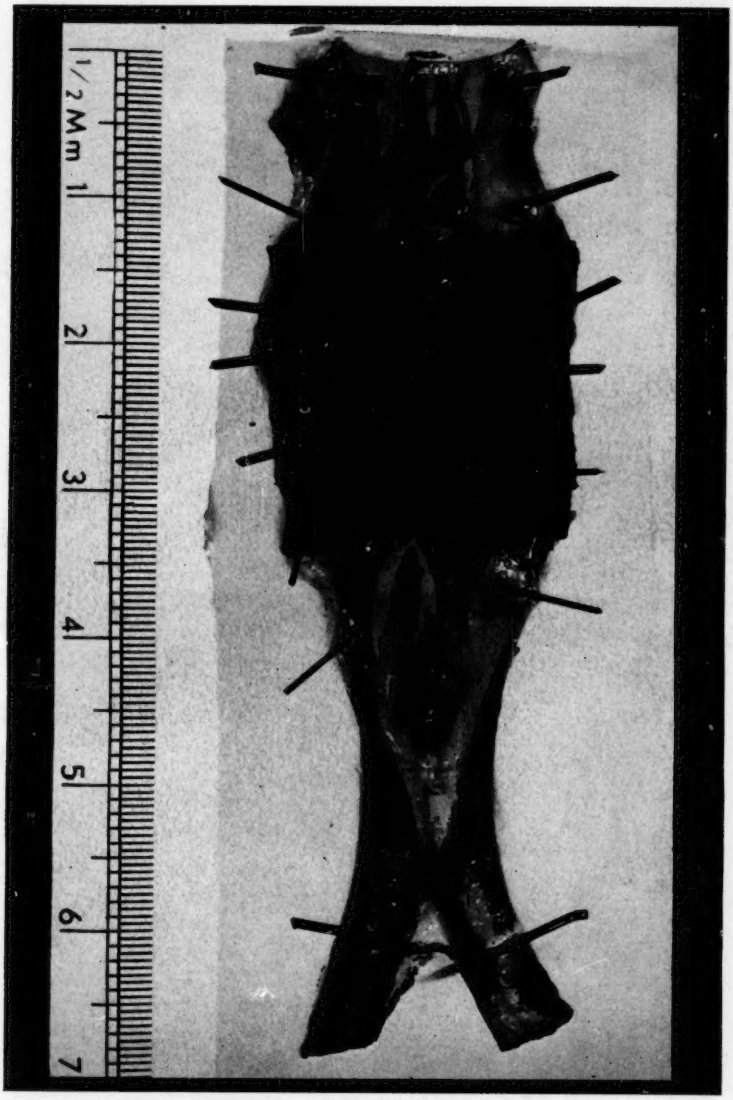
FIG. 17.—Electron micrograph of a section through the superficial part of the pseudointima. E: endothelial cell. The subendothelial tissue consists of elastic fibres (EL) which are very electron dense, collagen fibres (COL) and cells which have the appearance of smooth muscle (SM). N: nucleus. Tissue treated with phosphotungstic acid before embedding in Araldite. ×5,000.

FIG. 18.—Electron micrograph of a section through the pseudointima showing parts of 2 smooth muscle cells cut longitudinally. They contain parallel fibres (myofibrils) and some small dark objects which are usually seen in smooth muscle. Endoplasmic reticulum (ER) is found near the poles of the nucleus (N). Tissue treated with phosphotungstic acid before embedding in Araldite. ×13,000.

FIG. 19.—Electron micrograph of a section through the pseudointima showing part of a smooth muscle cell. Collagen fibres are seen near the end of the cell (lowest arrow). Other structures are labelled as for Fig. 18. Tissue treated with phosphotungstic acid before embedding in Araldite. ×13,000.

FIG. 20.—Electron micrograph of a section through the pseudointima showing fibrous components. The dark material to the left of the field is elastic tissue (EL). Most of the other structures are collagen fibres (COL) whose characteristic banded appearance can be made out. The fibrillary material (FF) in the upper right hand corner of the field has not been identified. It may well be different from the fine fibres illustrated in Figs. 10 and 21. Tissue treated with phosphotungstic acid before embedding in Araldite. ×92,000

FIG. 21.—Electron micrograph of a section passing through part of an endothelial cell lining the pseudointima. Fine fibres (FF) apparently lying immediately beneath the endothelium are seen clearly (cf. Fig. 10). Other structures as in Fig. 10. Tissue treated with phosphotungstic acid before embedding in Araldite. ×28,000.



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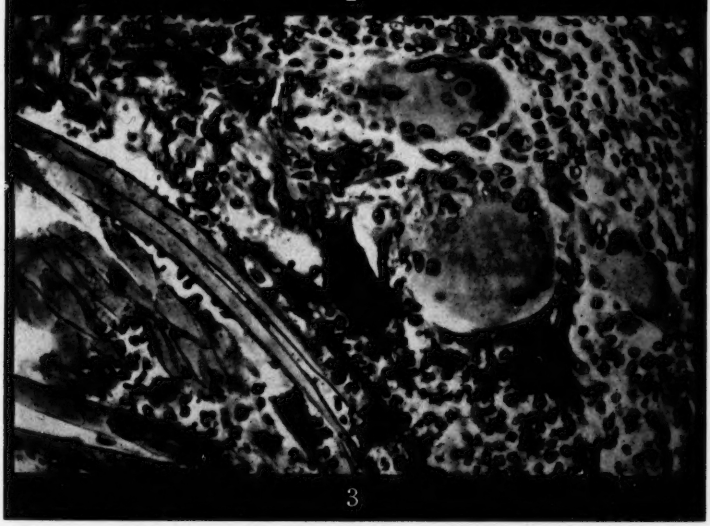

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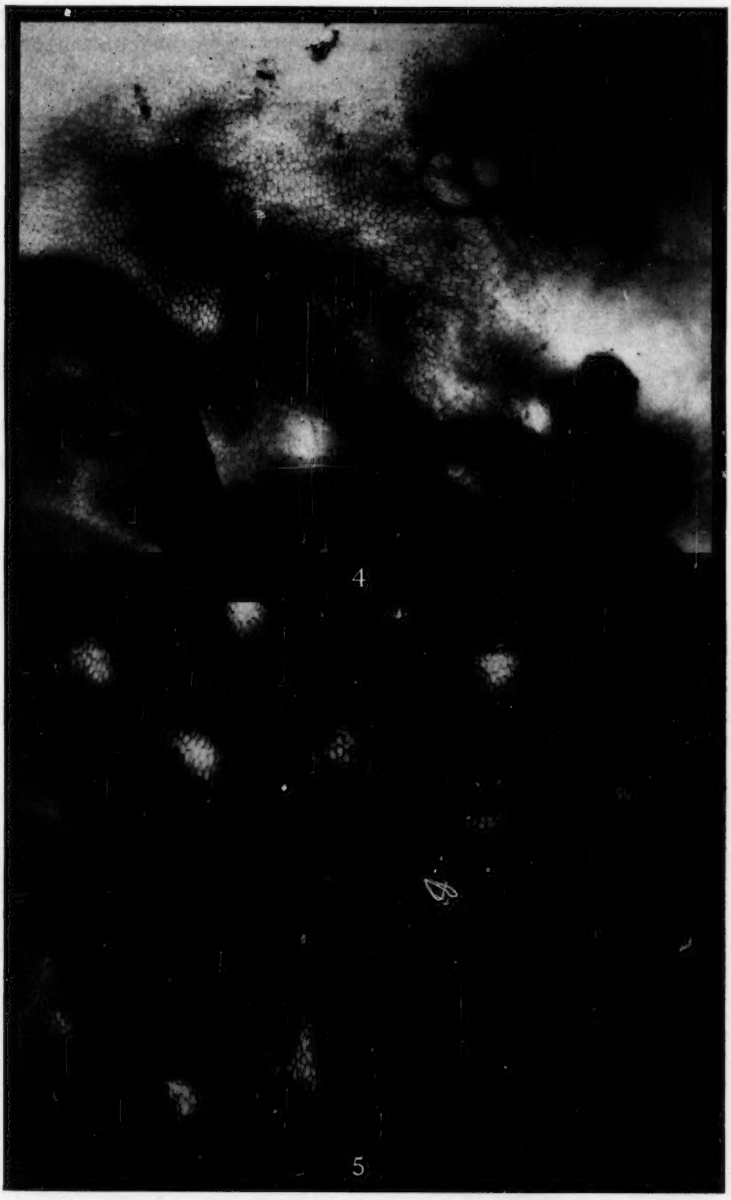

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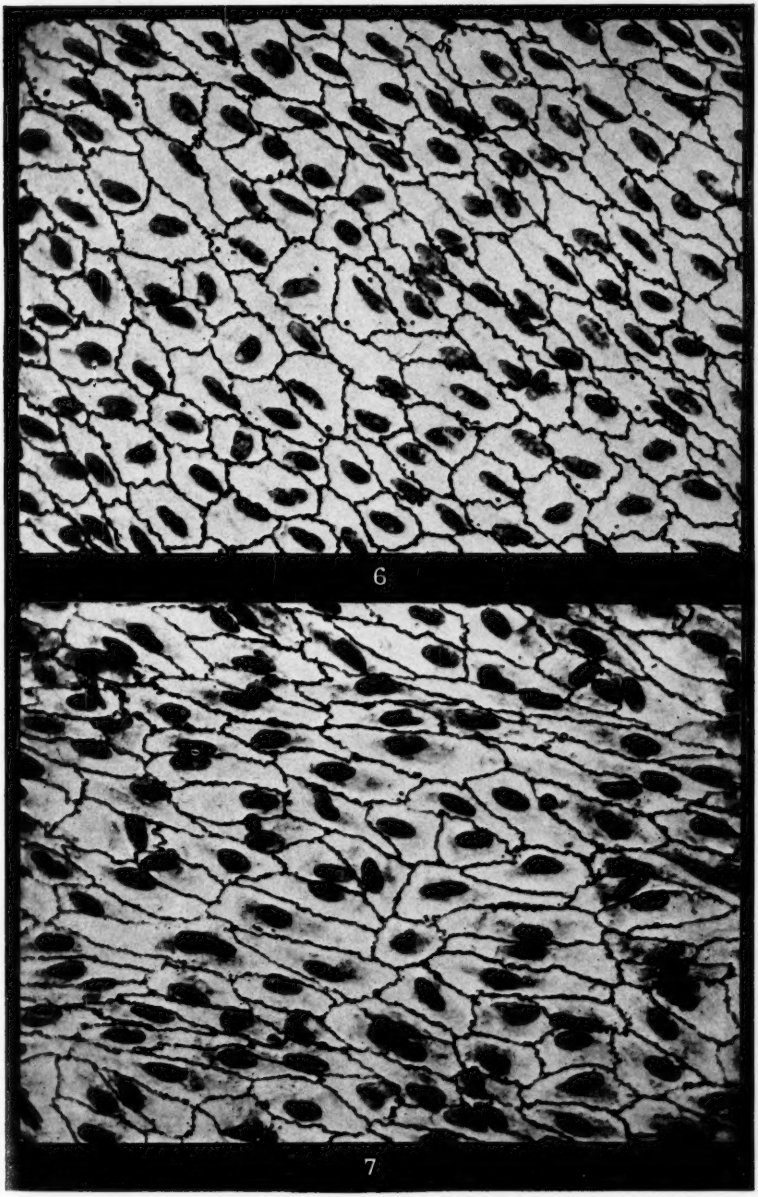

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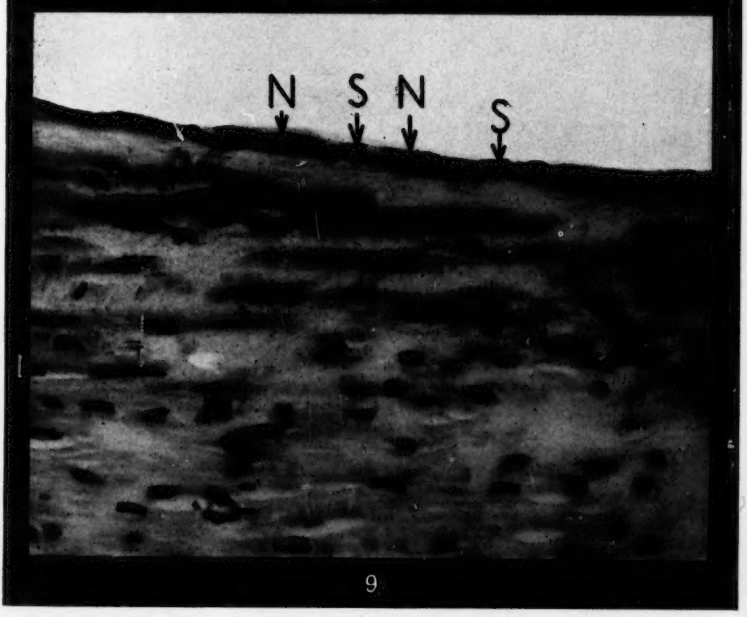

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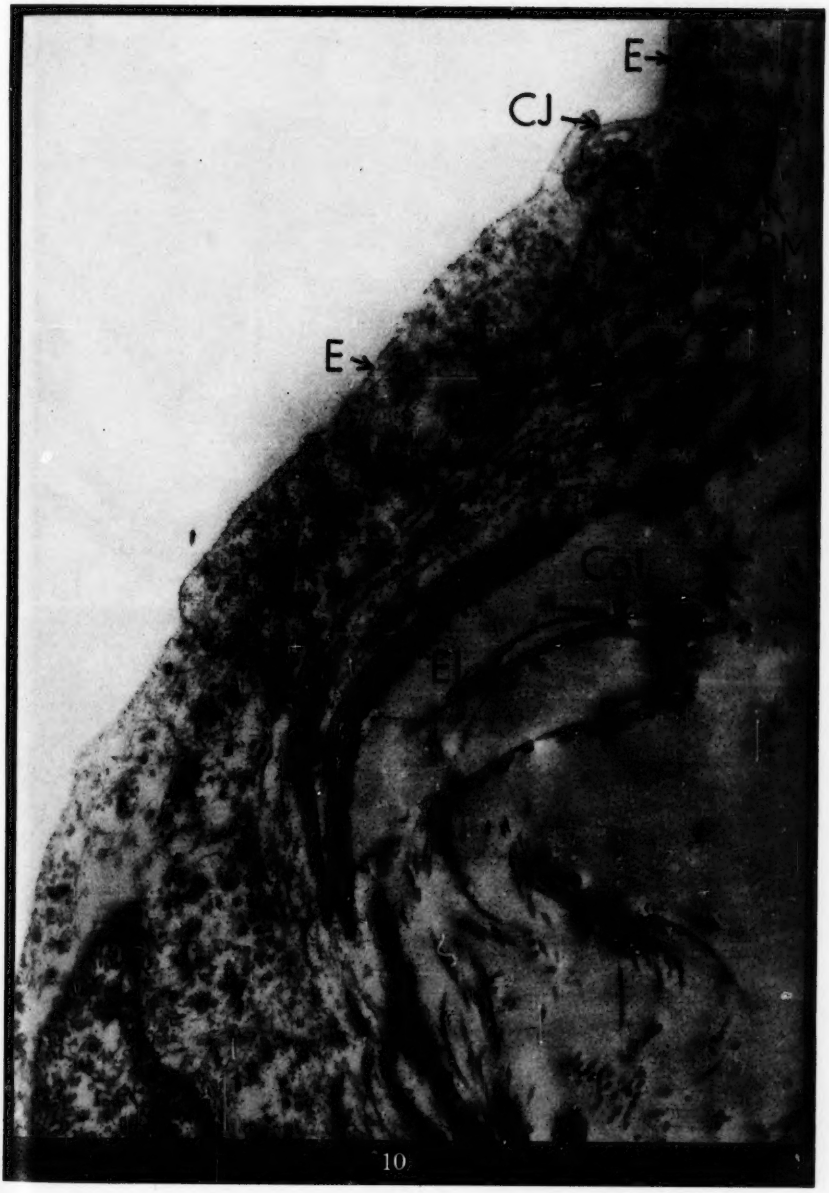

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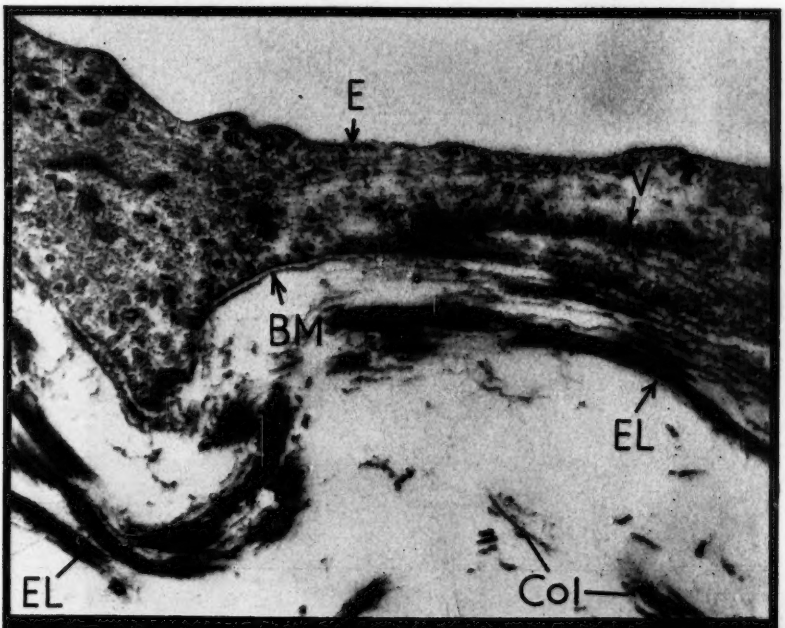

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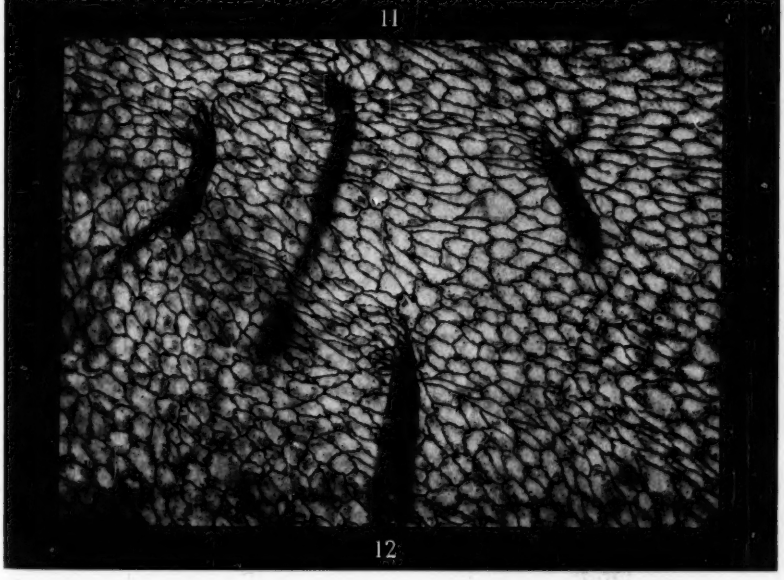

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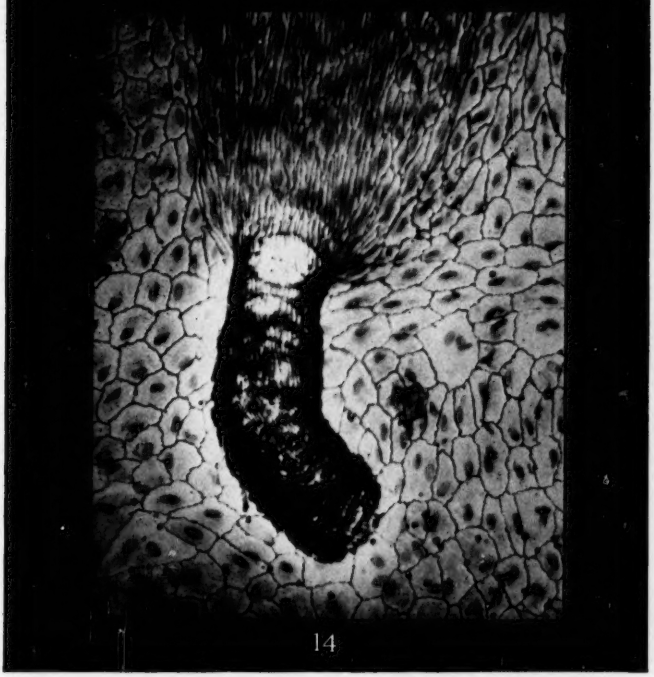

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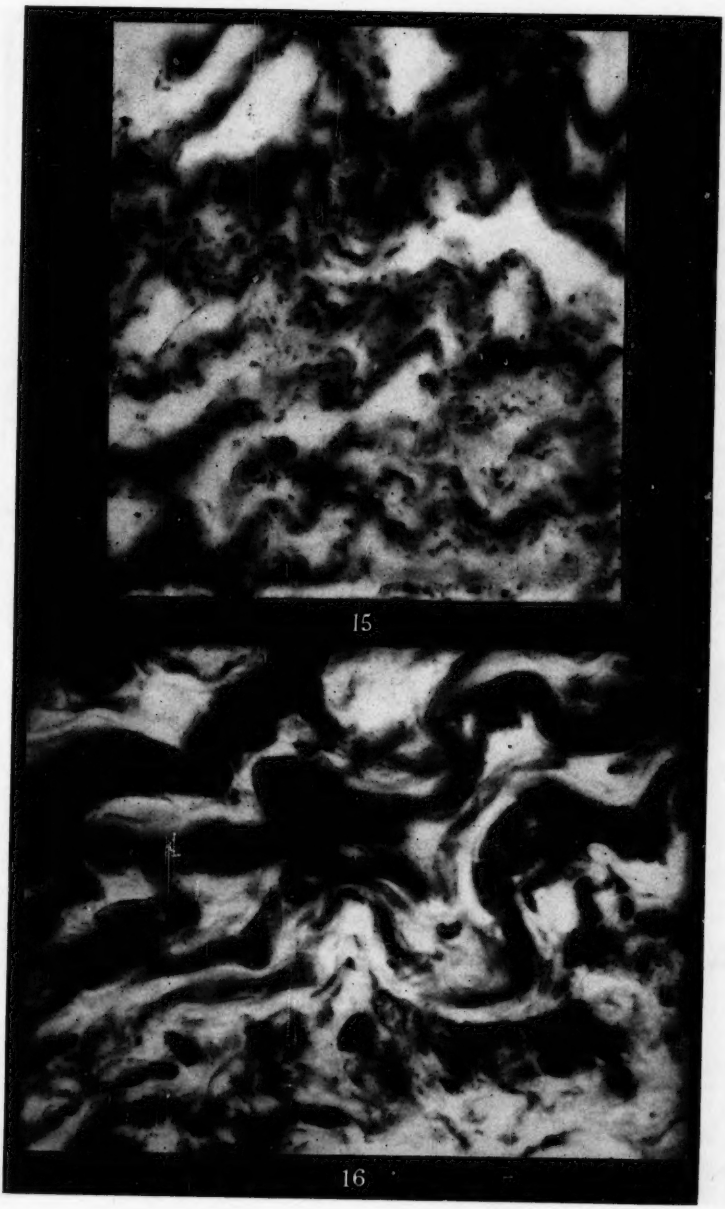

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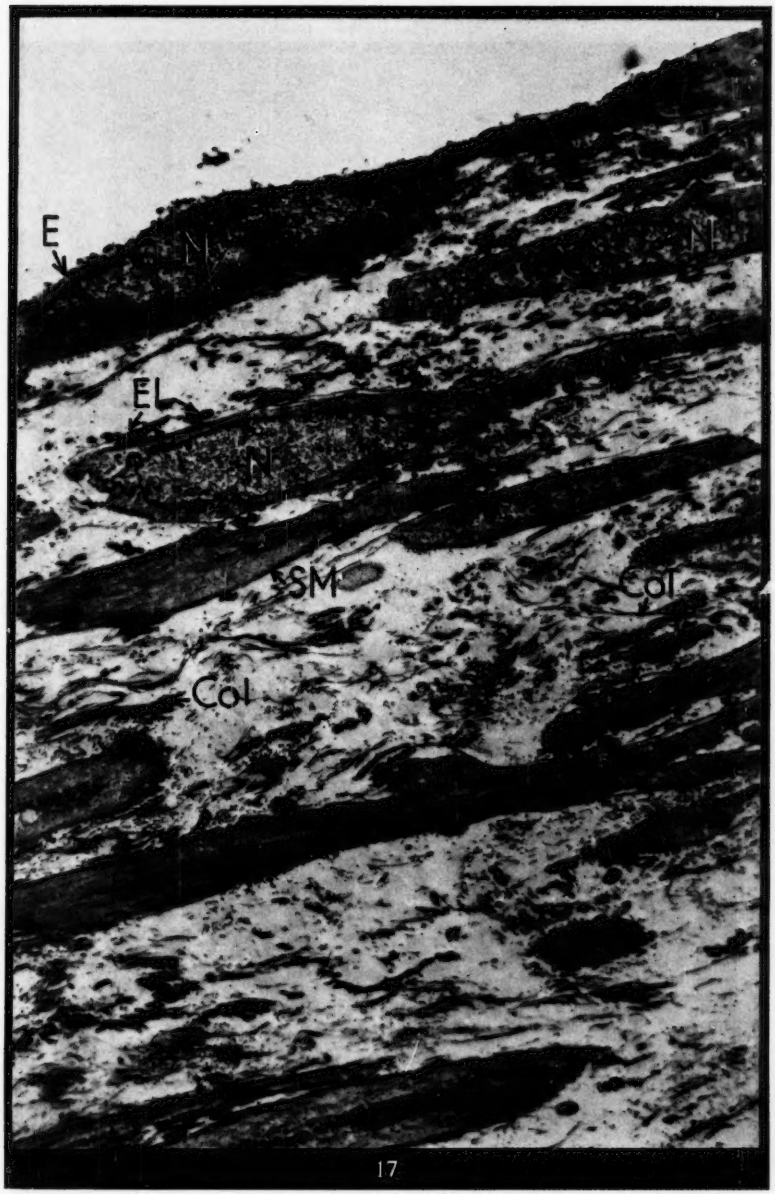

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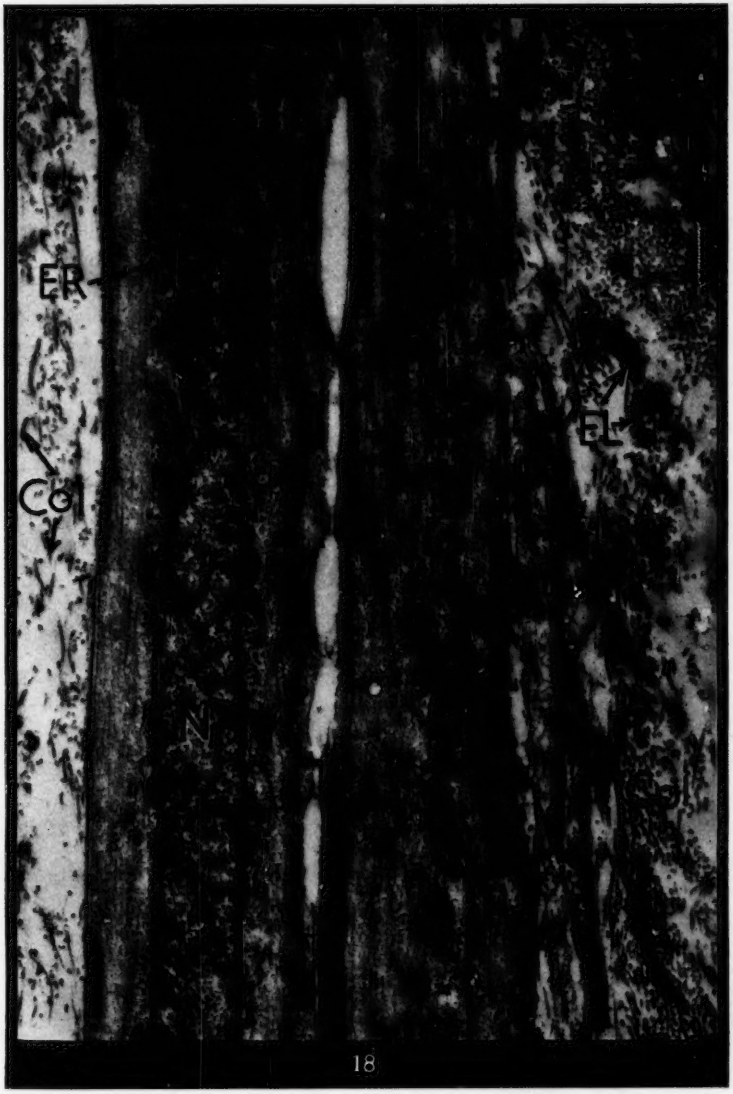

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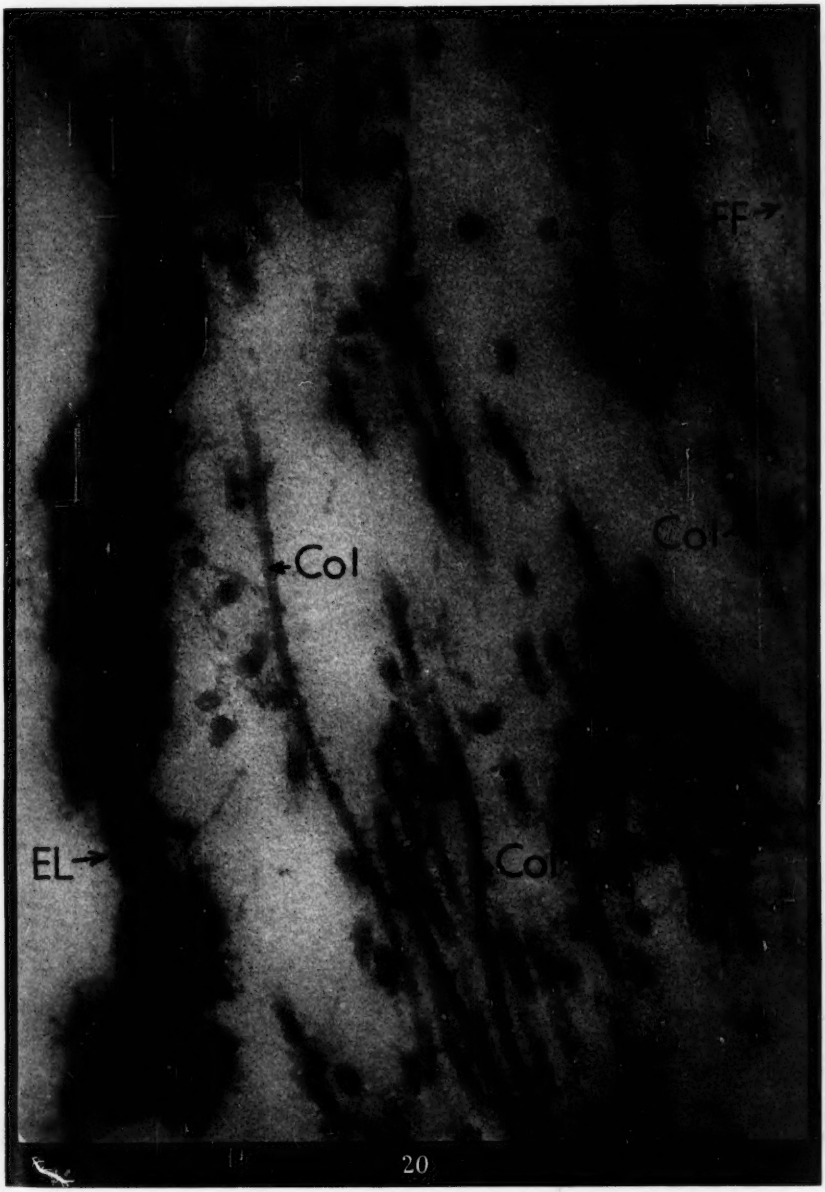

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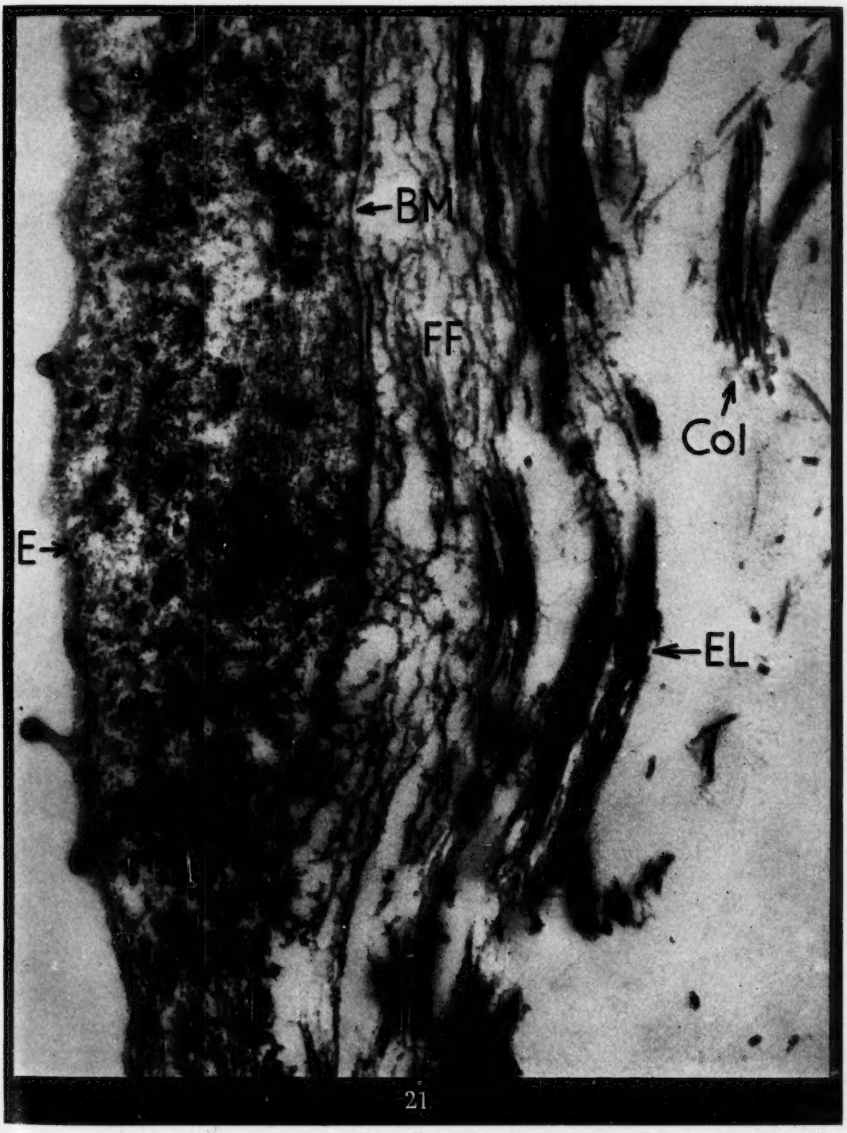

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they appear to be and that the whole question of intimal vascularization from the lumen in relation to atherosclerosis perhaps deserves more attention than it has so far received.

The tissue between the endothelium and the cloth

The only type of cell in this region which can be identified with confidence is smooth muscle, and this is the predominant cell type. From the fact that collagen is present in abundance it is reasonable to suppose that fibroblasts are also present, though no fibroblasts could be identified with certainty in any of our preparations. Our difficulty in recognizing fibroblasts may be due to the long sojourn of the tissue in 70 per cent alcohol.

The occurrence of smooth muscle cells in the tissue immediately underlying the endothelium in large arteries has in the past not been generally recognized, either in normal or in diseased arteries. The conventional view of the structure of the tissue immediately beneath the endothelium is that expressed by Maximov and Bloom (1952) who stated that " it consists of a few interlacing fibres and fibroblasts ". Smooth muscle cells, they believed, were only found in the deeper parts of the intima. Nevertheless Florey (1960*b*) was able by electron microscopy to demonstrate cells with the appearance of smooth muscle immediately beneath the endothelium in the thoracic aorta of a normal rabbit.

Recent investigations of the nature of the cells in the thickened intima of ligated vessels has emphasized the importance of smooth muscle. It has been generally supposed that the vessels were obliterated by endothelial proliferation (see Mehrotra, 1953). Recently Buck (1961) has reinvestigated the problem by electron microscopy and shown that the cells in the intimal thickenings in ligated vessels are mainly smooth muscle.

Yet another situation in which smooth muscle seems to be more important than was previously realized is in the fibrous plaques in human atherosclerosis. The predominant cell type in these lesions was supposed by Crawford and Levene (1952) and by Prior and Jones (1952) to be the fibroblast, but More, Movat and Haust (1957) and Haust, More and Movat (1959, 1960) have produced apparently convincing evidence that most of these cells are in fact smooth muscle cells, although their methods of investigation did not include electron microscopy.

There have been many histological studies of the structure of the intimal thickenings of the aorta in rabbits fed cholesterol, but it is only recently that Parker (1960), using the electron microscope, has demonstrated clearly that these thickenings contain smooth muscle. Elongated cells in these lesions have been illustrated before (*e.g.* Poole and Florey, 1958, Fig. 12) but their nature has hitherto been uncertain.

The present investigation shows that in a fourth abnormal situation, the pseudointima lining fabric grafts, the predominant subendothelial cell is the smooth muscle cell.

These smooth muscle cells may be of importance in the pathogenesis of atherosclerosis. Geer, McGill, Strong and Holman (1960) have shown that in human atherosclerosis studied by electron microscopy the earliest detectable lesions are fat droplets in smooth muscle and endothelium.

Nothing was seen which might indicate the origin of the smooth muscle cells in the graft ; they may have arisen from muscle cells in the cut ends of the aorta, but the possibility that they migrated through the interstices of the knitted fabric

cannot be excluded. Haust *et al.*, (1960) in their studies on human fibrous plaques suggested that endothelium turns into smooth muscle. Endothelium and smooth muscle are distinguishable by the electron microscope and no intermediate stages between these 2 types of cell were seen in the present studies.

The fibrous components of the pseudointima comprise collagen and elastin and certain fine fibres whose nature is uncertain, which occur just beneath the endothelium in most of the areas examined. Other fine fibres (which may be different from those just mentioned) were seen in the deeper layers (Fig. 20). The collagen and elastic fibres are well separated and probably lie in a matrix composed of material which does not react with osmium tetroxide or with phosphotungstic acid.

SUMMARY

Knitted polyethylene glycol terephthalate (" Dacron ") grafts were inserted into the lower abdominal aorta in 8 baboons which were killed and examined 10 wks. later. As found by previous workers in other species, a substantial layer of tissue, the pseudointima, had formed inside the graft. The graft was completely lined by endothelium which was identified by its appearance in surface preparations and in sections examined by electron microscopy. The pseudointima contained many elongated cells most of which were smooth muscle cells. In places, foreign body giant cells and polymorphonuclear leucocytes were seen. Fibrous components of the pseudointima comprised collagen, elastin and some unidentified fine fibres, some of which have the staining properties of reticulin. Small vessels, with internal diameters ranging from approximately 300 μ down to approximately 10 μ, arise from the lumen of the graft.

Major-General Elbert DeCoursey, Director of the Southwest Foundation for Research and Education, gave every possible encouragement to this investigation. The animals at operation were anaesthetized by Dr. Georgiana Greer and surgical assistance was provided by Mr. Tom Nthumbi Nzoga. In San Antonio H. W. F. and J. C. F. P. were assisted by various members of the technical staff of the Southwest Foundation for Research and Education, particularly by Mrs. Nancy Werthessen and Mr. Arthur Rodriguez. Some of the photomicrographs of Häutchen preparations were taken in New Orleans by Professor Henry C. McGill, Jr., and Mr. Eugene S. Black of the Pathology Department of Louisiana State University. In Oxford, histological sections were cut and stained by Frl. Rose Niederberger and Mr. D. W. Jerrome ; Mr. Jerrome also prepared specimens for electron microscopy ; photomicrographs were taken by Mr. S. A. Buckingham, and Frl. Christa Torriano assisted in the preparation of photographic prints. Dr. J. E. French and Dr. Margaret Jennings kindly read the manuscript and made valuable suggestions.

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THE EFFECTS OF DEPRIVATION OF PORTAL BLOOD ON THE LIVER AND ITS INFLUENCE ON CARBON TETRACHLORIDE LIVER INJURY IN THE RAT

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THE liver injury produced by carbon tetrachloride has been studied by many workers, notably Cameron and Karunaratne (1936) who described the characteristic pattern of necrosis and its mode of resolution. The factors causing this necrosis have been investigated with emphasis on the intra-hepatic anatomy of liver blood flow (Himsworth, 1950; Daniel, Prichard and Reynell, 1952). Although there is no clear relationship between liver blood flow and the development of centrilobular necrosis, it has been speculated that the portal blood supply exerts a protective effect in toxic or infective processes involving the liver. The portal blood is thought to bring to the liver nutrient substances which may influence the degree of damage sustained by it. The greater damage sometimes seen in the left lobe of the human liver in fatal cases of viral hepatitis has been attributed to the " streams " of blood in the portal vein (Copher and Dick, 1928) by which protective substances from the small intestine are carried mainly to the right lobe.

It would follow that complete absence of portal blood might greatly influence the extent of the necrosis produced by carbon tetrachloride.

There has also been some conflict of opinion over the relationship of the portal blood supply and regeneration of the liver. On the one hand it was concluded (Mann, Fishback, Gay and Green, 1931; Mann, 1940) that regeneration of the liver after partial hepatectomy is dependent on an intact portal blood supply, whereas more recent work (Weinbren, 1955) showed that regeneration will occur in liver tissue deprived of portal blood. These workers did not extend their reported observations to the effect of portal blood on the restoration of the liver after toxic damage. This paper reports, therefore, the effects of depriving part of the rat liver of its portal blood supply, and the influence which this deprivation exerts on the size of the necrotic areas induced by carbon tetrachloride and on their subsequent resolution.

MATERIAL AND METHODS

Male and female albino rats weighing 150–250 g. and bred in this laboratory were fed " diet 41 " cubes and water *ad libitum*.

Operative procedures were carried out under ether anaesthesia.

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The following groups of animals were used :—

Group I. Ligation of the portal vein branches to the anterior lobes

The branches of the portal veins to the left and median lobes (Greene, 1955) were ligated. One ligature occluded the vein supplying the left lobe and the left half of the median lobe, and another ligature was placed around the branch supplying the right half of the median lobe. The abdomen was then closed in layers. There were 36 rats (15 female and 21 male) in this group, and animals were killed at varying intervals up to 28 days post-operatively.

Group II. Ligation of branches of portal vein and injection of carbon tetrachloride

The procedure described for Group I was followed by subcutaneous injection in the flank of carbon tetrachloride 0·1 ml./100 g. body weight. The injection was given after recovery from anaesthesia. These animals were killed at varying times up to 15 days post-operatively. There were 17 rats (8 male and 9 female) in this group.

Group III. Ligation of one branch of the portal vein followed by injection of carbon tetrachloride

A simpler procedure was used in this group of 14 male rats. Only one portal vessel supplying the left lobe and left half of the median lobe was tied off. This vessel is more easily ligated than that supplying the right half of the median lobe, and the duration of the operation is much shorter than when both vessels are tied. Operative shock with its disturbances of systemic and portal blood flow is reduced to a minimum. Carbon tetrachloride was injected 24 hr. post-operatively, when recovery was more complete than when injected in Group II. These animals were killed 24 hr. after the administration of carbon tetrachloride.

Group IV. Control group. Laparotomy and injection of carbon tetrachloride

Laparotomy and subsequent injection of carbon tetrachloride was done in 12 male and 12 female rats, which were killed at varying times up to 18 days later.

Animals were killed by a standard procedure. Under ether anaesthesia a small quantity of methylene blue was injected into the spleen, and any animals showing incomplete portal obstruction to the anterior lobes, or infarction, were not used in the experiment. The hepatic veins, vena cava and aorta were then cut across immediately below the diaphragm and the livers removed. After blotting away excess blood the anterior and posterior liver lobes were weighed separately. Tissues were embedded in paraffin and the usual methods used for microscopical examination.

Measurement of the extent of necrosis

The assessment of the amount of necrosis present 24 hr. after the injection of carbon tetrachloride was carried out by a method similar to that described by Ogilvie (1937). Six consecutive fields from individual anterior and posterior lobes were projected onto fine quality tracing paper. The areas of necrotic and healthy cells were outlined and the paper cut up. The papers representing these areas of the liver were then weighed separately and the necrotic area expressed as a percentage of the total weight. Four livers of Group II, and 5 livers of Group III were assessed this way.

RESULTS

Group I

When the portal veins to the anterior lobes are ligated, the posterior lobes rapidly increase in weight from approximately 30 per cent of the total liver weight in the normal, to 63 per cent after 3 days, 75 per cent after 6 days and 87 per cent after 28 days (Fig. 1, Table I).

The anterior lobes become atrophic and the relative proportions of the liver become stable at about 18 days after portal vein ligation. They become progressively more deeply brown in colour, and macroscopically give a strongly positive prussian blue reaction.

TABLE I.—*The Relative Weights of Anterior and Posterior Lobes of the Liver after Ligation of the Portal Vein Branches to the Anterior Lobes*

Number of rats	Days after ligation	Mean wt. of posterior lobes (per cent total liver wt.)	Range	S.D.
Normal controls				
10 females	—	31·4	29·5–34·7	±1·6
11 males	—	33·8	30·0–37·0	±1·9
Group I				
8	3	63	56–65	±3
7	6	75	66–80	±4·9
4	18	89	88–89	±1·5
11	28	87	84–92	±2·3

The microscopic appearances of the anterior lobes reflect this progressive atrophy, in that the hepatic cells become smaller, more fibrous tissue is seen and vessel walls and liver capsule are thrown into sharp folds. Kupffer cells become prominent, and by the third post-operative day show a faint blue flush with the prussian blue reaction. Later coarsely granular haemosiderin is present in Kupffer cells which become aggregated into masses related to portal tracts, hepatic vein radicles and along fibrous septa (Fig. 2). The pigment does not give a reaction with the long Ziehl-Neelsen method for acid fast material. No pigment is seen in the parenchymal cells. More cross-sections of bile ducts than normal are seen in the portal tracts, and mitoses occur in the bile duct epithelium.

The changes in the posterior lobe are characteristic of regenerating liver. Immediately after the portal veins to the anterior lobes are obstructed, the posterior lobes become bright red, and the lobular pattern is accentuated. Later, the posterior lobes become enlarged and tawny-yellow in colour. Microscopically cells and nuclei vary greatly in size during the period of rapid growth. Mitoses are few at 24 hr., but abundant from the 3rd to the 12th day. By the 18th day few mitoses are present, and at the 28th day are very rare. Cell and nuclear size is also more uniform at the 28th day, but the lobules are still enlarged. During this period of regeneration there is a marked difference between the appearance of the cells in the periportal area and that of cells in the region of the hepatic vein radicle. The periportal cells are on the whole strikingly larger with marked cytoplasmic basophilia, and also marked variability in nuclear and cell size. Mitoses are more numerous in the periportal region. Cells are on the whole smaller in the region of the hepatic vein radicles (Fig. 3).

Groups II, III, and IV

The qualitative changes produced by carbon tetrachloride are present in both atrophied anterior lobe and regenerating posterior lobe. The ballooned hydropic cells in the anterior lobes are smaller than those of the posterior lobe (Figs. 4 and 5).

The results of the quantitative assessment of the amount of necrosis present 24 hr. after carbon tetrachloride show that in 3 out of 9 animals measured the necrosis in the lobe deprived of portal blood is greater than in the lobe with an intact blood supply (Table II), and this in a degree which cannot be regarded as significant when using the present method of assessment. In the other animals

TABLE II.—*The Extent of Liver Necrosis due to Carbon Tetrachloride in Portal-deprived and Regenerating Lobes*

	Per cent necrosis in anterior (atrophic) lobes	Per cent necrosis in posterior (regenerating) lobes
Group II		
rat (a)	28	51
(b)	26	28
(c)	42	38
(d)	31	30
Group III		
rat (e)	28	46
(f)	15	40
(g)	18	30
(h)	21	17
(i)	37	50

the necrosis is more extensive in the regenerating lobe. It seems clear that the absence of portal blood has not made the necrosis more severe. Both the atrophied and the regenerating lobes show the same type of inflammatory reaction in response to carbon tetrachloride, and repair and restoration of the lobular architecture occurs at the same rate in both. Mitoses are seen in the atrophic anterior lobe during the period of repair. The rate of repair is similar in animals of Groups II, III, and IV. No sex differences in the reaction to carbon tetrachloride are seen in these experiments.

DISCUSSION

After interruption of the portal blood supply to the two anterior lobes, these remain with only arterial blood, while the pathway for all the portal blood is now through the posterior lobes. It is not known whether the total amount of blood-flow in the hepatic artery or in the portal vein is altered.

The method used for measuring the extent of necrosis due to carbon tetrachloride is semi-quantitative only. No statistical conclusions have been based on the findings, and the method has been used merely to indicate that microscopic

EXPLANATION OF PLATE

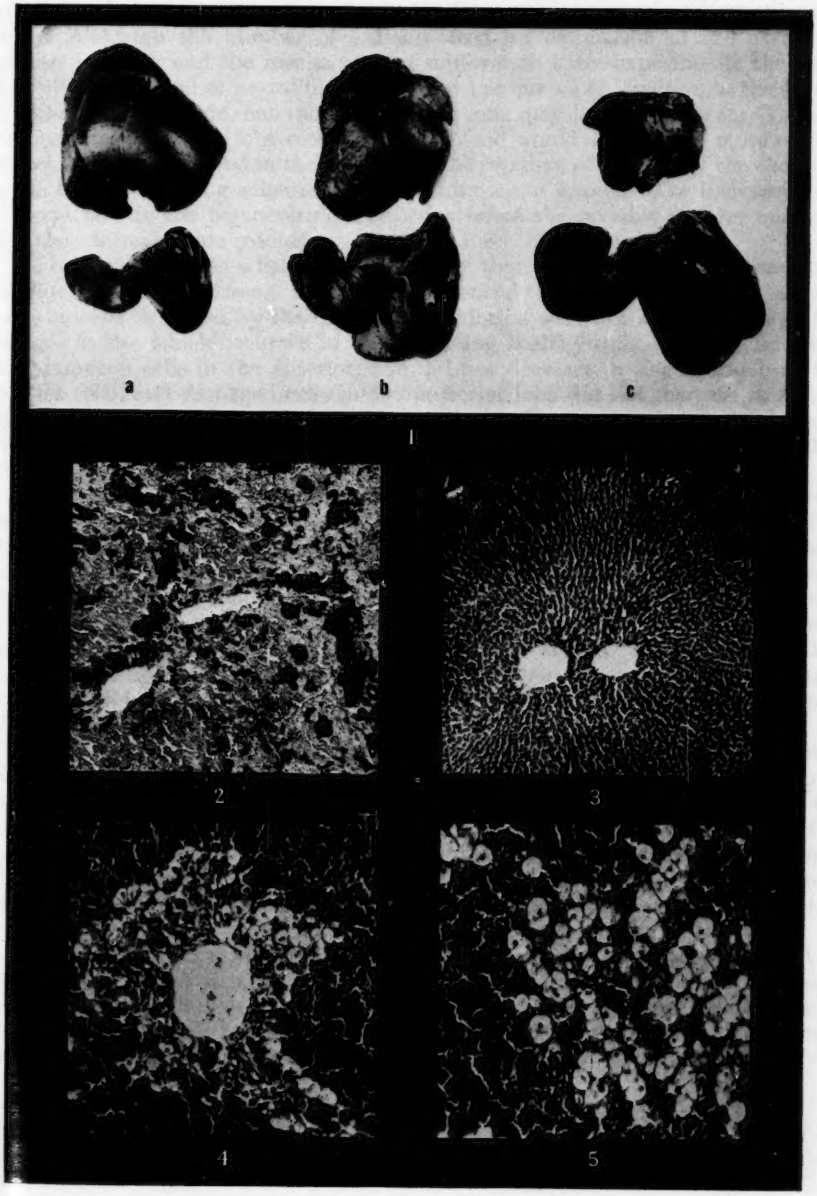
FIG. 1.—The anterior lobes above and posterior lobes below of the livers of (a) normal rat, (b) 3 days and (c) 18 days after the portal blood supply to the anterior lobes had been interrupted. There has been progressive atrophy of the anterior lobes and hypertrophy of the posterior lobes.

FIG. 2.—Section of atrophied anterior lobe 28 days after ligation of its portal vein branch. Haemosiderin is present in Kupffer cells which lie in masses adjacent to hepatic vein radicles and portal tracts. (Prussian blue reaction. ×110.)

FIG. 3.—Section of posterior lobe 12 days after ligation of portal vein branches to anterior lobes. The cells in the periportal regions are larger than those in the region of hepatic vein radicles. (H. and E. ×110.)

FIG. 4.—Section of anterior lobe 48 hr. after interruption of its portal blood supply, and 24 hr. after subcutaneous injection of carbon tetrachloride. The characteristic hydropic cells which are present are smaller than those found in unoperated livers of the control group. (H. and E. ×110.)

FIG. 5.—Section of the hypertrophied posterior lobe from the same liver as Fig. 4. The hydropic cells are much larger than those in the anterior lobe as well as those of the control group. (H. and E. ×110.)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differences between anterior and posterior lobes may be put onto an objective basis. Although the number of animals used for estimation of the extent of necrosis is small, and the results are not uniform, in these experiments the parts of the liver deprived of portal blood have not become more sensitive to the effects of carbon tetrachloride, but the regenerating lobe may have become so. There is the possibility that the lobe receiving more blood would also receive more carbon tetrachloride transported in the blood, but the rapidity of fixation of the chemical in the tissues following subcutaneous injection is not known. The increased rate of blood flow in the regenerating lobes may offset the possibly greater quantity of carbon tetrachloride reaching it.

The question arises whether factors other than the amount of carbon tetrachloride reaching the tissue, could have influenced the results. It may be argued that the cells damaged by the carbon tetrachloride were not able to undergo the changes in size which occurred in the remaining healthy cells. This would mean that damaged cells in the anterior lobe did not decrease in size as much as the healthy cells, and damaged cells in the posterior lobe did not increase in size as much as the other cells undergoing hypertrophy. However, this is not in accord with the observations of the alterations in size of the necrotic cells (Fig. 4 and 5) and this artefact has probably not occurred.

Rous and Larimore (1920) showed that deprivation of portal blood from liver tissue in the rabbit caused atrophy. In the present experiments the atrophy is regularly accompanied by the accumulation of haemosiderin in Kupffer cells, but not in parenchymal cells. No haemosiderin is seen in the livers with an intact portal blood supply, whether following necrosis or not, and whether in normal or regenerating liver. All animals were on the same diet, adequate in protein and water, and the food containers were not made of iron. It seems likely that the accumulation of haemosiderin in the Kupffer cells is a result of interruption of the portal blood supply, and may therefore be related to the liver cell atrophy or the presence of only arterial blood supply.

The very large size of the periportal cells of the regenerating lobes compared with the cells in the region of the hepatic vein radicles, which is observed up to the 18th postoperative day, seems to indicate a greater growth activity in the periportal region. The rate of regeneration of the posterior lobes is slower after ligation of portal veins to the anterior lobes than it is after partial hepatectomy. This may explain why the contrast in cell sizes is seen more clearly in the present animals than after partial hepatectomy. Mall (1906) put forward the concept of a liver lobule with its centre based on the portal tract and its blood vessels. If regeneration is an exaggeration of normal growth, the greater growth activity in the periportal area seen here may indicate that normal growth and perhaps differentiation, although slow, tends to occur from the periportal cells towards the hepatic vein radicle. The " limiting plate " (Elias, 1949) may actually represent a basal layer of liver cells.

SUMMARY

The anterior lobes of the rat liver have been deprived of portal blood by ligation of branches of the portal vein, and the changes occurring in the liver have been described.

The effect of the deprivation of portal blood on the development and resolution of liver damage produced by carbon tetrachloride was studied.

It was found that the lack of portal blood did not increase the extent of necrosis caused by carbon tetrachloride, nor was the process of healing impaired.

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THE RELATION OF THE TOXIC AGENT TO THE SUBUNITS OF INFLUENZA VIRUS PARTICLES

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IT has been reported that the toxic property of influenza virus could not be separated from the infective property by such means as differential centrifugation and adsorption onto and elution from red cells, while with certain inactivating agents the infective property was lost at a faster rate than the toxic property (Henle and Henle, 1946, 1947). In an earlier paper we have reported that the toxic effect of influenza virus, PR8 strain, given intravenously to rabbits, was rather strengthened by sonic vibration in spite of the loss of most of the infectivity (Kato and Hara, 1961). In later studies it has been confirmed by electron microscopy and ultracentrifugation that the influenza virus particle is disintegrated nearly completely into its subunits by sonic vibration for 1 hr. Ether has been shown to disintegrate influenza virus particles (Hoyle, 1952; Hoyle, Reed and Astbury, 1953; Schäfer and Zillig, 1954), but it has been found in our studies that this treatment completely destroys virus toxicity. Thus, sonic vibration seems to be the only useful way to disintegrate influenza virus particles without destroying their toxicity. Experiments have therefore been made, using sonic vibration, to examine the distribution of the toxic agent in the subunits obtained after splitting influenza virus particles. The present paper describes these experiments.

MATERIALS AND METHODS

Preparation of virus suspensions

The PR8 strain of influenza A virus was used. The procedures for the preparation of partly purified elementary particle (EP) suspensions were identical with those already described (Kato and Hara, 1961). In many cases these EP suspensions were further purified by 1 of the 2 following procedures.

Red cell method.—To a suspension chilled to 4° were added 5 per cent packed, washed chicken red cells. The red cell-virus mixture was kept at 4° for 30 min. The red cells were sedimented by centrifugation in the cold, washed once with ice-cold 0·01 M-phosphate buffered saline at pH 7·2 (PBS), resuspended in the original volume of PBS, and incubated for 90 min. in a 37° water bath. The red cells were removed by centrifugation and the supernatant containing the eluted virus constituted the purified elementary particle suspension by the red cell method (EP–RC).

Aluminium phosphate column method.—The aluminium phosphate column method of Miller and Shlesinger (1955) as modified by Frommhagen and Knight (1959) was used. A virus suspension was diluted appropriately before being introduced into the column. The eluates were preliminarily centrifuged at 3,000 r.p.m. for 10 min., to remove the contaminating column material, and then centrifuged at 44,000 *g* for 30 min. The viral pellets were resuspended in PBS to the original volume of the virus suspension. This suspension was finally centrifuged at 3,000 r.p.m. for 15 min. and the supernatant constituted the purified elementary particle suspension by the column method (EP–C).

Treatment of virus suspensions with sonic vibration

This procedure was as already described (Kato and Hara, 1961). Sonic vibration lasted for 1 hr. except in certain experiments.

Treatment of virus suspensions with ether

Virus suspensions were mixed with ether at room temperature for 2 hr. by the method standardized by Lief and Henle (1956*a*).

Separation of the haemagglutinin and S antigen fractions

The haemagglutinin (HA) of sonically vibrated virus suspensions was separated from S antigen by adsorption onto chicken red cells. A modification of the procedure described by Lief and Henle (1956*a*) was used. The suspensions were chilled to 4° and absorbed with 5 per cent packed, washed chicken red cells for 30 min. at 4°. The red cells were sedimented by centrifugation in the cold, washed once with ice-cold PBS, resuspended in the original volume of PBS and incubated for 90 min. in a 37° water bath. The eluates contained the HA. The supernatant after absorption with red cells was further absorbed twice with 10 per cent red cells to remove the HA completely. The absorbed supernatant fluid contained the S antigen.

Haemagglutinin (HA) and infectivity titrations

These were identical with those already described (Kato and Hara, 1961). HA titrations were usually made with chicken red cells. In certain experiments guinea-pig red cells were also used. Infectivity titres were calculated in terms of EID_{50} per ml. according to the method of Reed and Muench (1938).

Complement fixation tests

For detection of S antigen, a complement fixation test was used. Anti-S sera were prepared by the method described by Lief, Fabiyi and Henle (1958). Guinea-pigs received active influenza virus (WS strain) intranasally and intraperitoneal booster doses of S antigen prepared from PR8 virus-infected allantoic fluid; the anti-S titre of their pooled sera was 1 : 128, and the anti-V (PR8) titre was 1 : 32. Anti-V antibodies in the serum were adsorbed by virus particles purified by 2 cycles of adsorption onto and elution from chicken red cells. Techniques of the complement fixation test were as described by Lief and Henle (1956*a*).

Electron microscopy and ultracentrifuge analyses

Electron micrographs of the elementary particle suspensions (EP–C, EP–RC), sonically vibrated suspensions, and of the HA and S fractions (at an instrumental magnification of 10,000) were taken with an Akashi model TRS–50 electron microscope. Drops of the preparations were placed on collodion-filmed grids, drained, air-dried and washed with distilled water to remove crystals of salts, dried again and shadowed with chromium.

Determinations of the ultracentrifugal behaviour of the elementary particle suspensions (EP–C, EP–RC), sonically vibrated suspensions, and of the HA and S fractions were made in the Spinco model E ultracentrifuge. The temperature at each determination was 20°. All preparations were suspended in 0·01 M-phosphate buffered physiological saline at pH 7·2. The sedimentation determinations were made on at least 3 different concentrations of each preparation. Sedimentation coefficients ($S_{20,w}$) were corrected to water as solvent at 20° and expressed as Svedberg units. The sedimentation rate of influenza virus has been shown by Lauffer (1946) to be concentration dependent. So, the sedimentation coefficients of the elementary particles and of the HA particles were determined by plotting the reciprocal of the sedimentation coefficients obtained for 3 preparations against the concentration at which they were run and extrapolation to infinite dilution.

Experimental animals and toxicity tests

Mice of the ddN strain weighing 20 to 25 g., supplied from the Central Laboratory of Experimental Animals (Tokyo), were used. The animals had been kept on the CE-2 solid feed and water in our laboratory for at least a week before the experiments. The toxicity of the various preparations was tested by the intravenous injection of twofold dilutions of the

preparations into groups of mice in a dose of 0·25 ml. per 10 g. of body weight. Mice which received an injection were examined for 10 days. Mice usually died within 5 days of the injection. Gross pathological changes were noted at necropsy. Delayed death caused by pulmonary consolidation in mice escaping early toxic death, as noted by Henle and Henle (1946), was not encountered. The LD_{50} for mice of the various preparations was calculated from the death rate of each group of mice according to the method of Reed and Muench (1938). In some of the animals the various organs were excised for histological examination. Sections of the tissue blocks were stained by haematoxylin and eosin.

RESULTS

Effect of ether and sonic vibration on the toxicity of influenza virus

A comparison of the effects of ether and sonic vibration on the toxicity of the virus was made. The results are shown in Table I. Ether treatment was for 2 hr.

TABLE I.—*Effect of Ether and Sonic Vibration on the Toxicity of Influenza Virus*

State of virus	Titres after treatment: log EID_{50}/ml.	Titres after treatment: HA/ml.	Dilution	Results of intravenous injection in mice (death rate)
EP untreated	9·1	1280	Undiluted	7/10
			Diluted 1 : 2	3/10
			„ 1 : 4	1/10
			„ 1 : 8	0/10
EP treated with ether	<2	640	Undiluted	0/10
			Diluted 1 : 2	0/10
			„ 1 : 4	0/10
			„ 1 : 8	0/10
EP treated with sonic vibration	4·6	2560	Undiluted	10/10
			Diluted 1 : 2	8/10
			„ 1 : 4	4/10
			„ 1 : 8	0/10

EP is the elementary particle suspension prepared by high-speed centrifugation.
Treatment with ether was performed for 2 hr., and sonic vibration for 1 hr.
LD_{50} for mice of each preparation based upon the titres of the original virus suspension is calculated from the data. The values are as follows:
EP : 215 HA units, $10^{8.32}$ EID_{50} per 10 g. of body weight.
EP treated with sonic vibration : 103 HA units, $10^{8.01}$ EID_{50} per 10 g. of body weight.

and sonic vibration for 1 hr. It is seen in the Table that virus toxicity was destroyed by ether and concurrently infectivity was lost nearly completely. On the other hand, sonic vibration strengthened toxicity about 2-fold in spite of the loss of most of the infectivity. In other experiments not presented here, toxicity was always destroyed nearly completely by ether treatment and strengthened up to 2 times following sonic vibration. A very small proportion of virus particles remained infective after sonic vibration. However, such a small amount of intact virus can never cause toxic death of mice, because the LD_{50} of intact virus particles for mice is much higher than this amount as shown in Table I.

The autopsy findings in mice which died from the intravenous injection of intact virus did not differ grossly or microscopically from those in mice which received sonically vibrated virus. The pathology of toxic death induced by the various preparations is described in a later section.

Effect of sonic vibration on the various properties of influenza virus

It was reported by Lief and Henle (1956*b*) that sonic vibration released some internal S antigen from influenza virus particles and that its release was not immediately accompanied by detectable loss of infectivity nor was a change noted in the degree of sedimentation of the virus particles by high-speed centrifugation. These results made it a possibility that the release of internal S occurred without the disintegration of the virus particles. In our studies, however, the release of internal S antigen was usually accompanied by a marked loss of infectivity. The discrepancy between their results and ours made the detailed reinvestigation of the effect of sonic vibration on the various properties of influenza virus particles necessary.

The elementary particle suspensions (EP–C, EP–RC) were exposed to sonic vibration for periods of up to 2 hr. The changes in HA titres and EID_{50} were examined. Haemagglutination tests were carried out with chicken (CHA) and guinea-pig red cells (GPHA). The results of a number of the experiments are shown in Tables II and III. It is seen in the Tables that following sonic vibration for ½ to 1 hr. in most cases HA titres of the elementary particle suspensions increased 4/3 to 4 times and the infectivity was always significantly lost. In some cases HA titres remained unaltered by 1 hr. sonic vibration, but even in

TABLE II.—*Effect of Sonic Vibration on the Haemagglutinin and Infectivity of Influenza Virus Suspension (EP–C)*

Experiment	Sonic vibration (hr.)	HA titre/ml. CHA	HA titre/ml. GPHA	log EID_{50}/ml.
1	0	3,840	1,920	—
	1/2	10,240	5,120	—
	1	10,240	5,120	—
2	0	5,120	2,560	—
	1/2	10,240	5,120	—
	1	10,240	5,120	—
3	0	5,120	2,560	10·2
	1/2	7,680	3,840	—
	1	10,240	5,120	6·2
4	0	5,120	2,560	10·5
	1/2	5,120	2,560	—
	1	10,240	5,120	7·6
5	0	10,240	5,120	—
	1/2	20,480	10,240	—
	1	5,120	2,560	—
6	0	20,480	10,240	10·7
	1/2	40,960	20,480	—
	1	20,480	10,240	6.0
7	0	20,480	—	10·7
	1	81.920	—	5.1
8	0	20,480	10,240	10.8
	1/2	20,480	10,240	—
	1	20,480	10,240	6·7
9	0	20,480	—	—
	1/2	40,960	—	—
	1	10,240	—	—
	1.1/2	5,120	—	—
10	0	40,960	—	10.9
	1/2	40,960	—	—
	1	81,920	—	6·8
	1.1/2	20,480	—	—
	2	10,240	—	5·0

TABLE III.—*Effect of Sonic Vibration on the Haemagglutinin and Infectivity of Influenza Virus Suspension (EP–RC)*

Experiment	Sonic vibration (hr.)	HA titre/ml. CHA	HA titre/ml. GPHA	log EID_{50}/ml.
1*	0	2,560	1,280	—
	1/2	5,120	2,560	—
	1	7,680	3,840	—
2	0	2,560	—	10·1
	1/2	5,120	—	—
	1	1,280	—	7·1
3	0	3,840	—	—
	1	5,120	—	—
4	0	5,120	—	—
	1	10,240	—	—
5	0	5,120	—	10·4
	1/2	5,120	—	—
	1	5,120	—	6·1
6*	0	5,120	2,560	10·3
	1	10,240	5,120	5·9
7	0	15,360	—	—
	1/2	20,480	—	—
	1	10,240	—	—
8	0	20,480	10,240	—
	1	20,480	10,240	—
	1·1/2	10,240	5,120	—
9	0	20,480	—	10·6
	1	40,960	—	7·2

* These EP–RC suspensions were prepared directly from the original infected allantoic fluids. The other EP–RC suspensions were prepared from the virus suspensions which had been concentrated by high-speed centrifugation.

such cases the infectivity titres were markedly reduced. The rise of HA titres and the decline of infectivity titres were seen in both EP–C and EP–RC suspensions. Following sonic vibration for longer periods of time than 1 hr. HA titres decreased. CHA titres were always twice GPHA titres, and the CHA/GPHA ratio was still unchanged after sonic vibration.

As described above, EP–C and EP–RC suspensions were usually prepared from the virus suspensions concentrated by high-speed centrifugation, and EP–C suspensions were again concentrated by a second high-speed centrifugation. Aggregation of the virus particles might have been caused by the high-speed centrifugation. If the rise of HA titres following sonic vibration had resulted from complete dispersion of such an aggregation of the virus particles, the infectivity titres should have been concomitantly enhanced. The data, however, show quite the reverse. Moreover, it is shown in Table III that sonic vibration still increased the HA titre of a non-sedimented purified virus suspension to a similar extent.

S antigen units in the elementary particle suspensions (EP–C, EP–RC), sonically vibrated suspensions, HA and S fractions separated from them were determined. Typical results are shown in Table IV. It is seen in the Table that the intact elementary particle suspensions were free of directly detectable S antigen, and following 1 hr. sonic vibration S antigen became detectable. The CHA/S ratio based upon CHA of the original elementary particle suspension was

TABLE IV.—*Separation of Haemagglutinin and S Antigen Fractions from Sonically Vibrated Suspensions*

Experiment	Preparation	CHA titre per ml.	S antigen units per ml.
1	EP–C	5,120	<10
	HAF	5,120	<10
	SF	<10	<10
	Sonically vibrated EP–C	10,240	80
	HAF	10,240	<10
	SF	<10	40
2	EP–C	10,240	<10
	HAF	10,240	<10
	SF	<10	<10
	Sonically vibrated EP–C	20,480	160
	HAF	20,480	<10
	SF	<10	160
3	EP–RC	5,120	<10
	HAF	5,120	<10
	SF	<10	<10
	Sonically vibrated EP–RC	5,120	40
	HAF	10,240	<10
	SF	<10	40
4	EP–RC	10,240	<10
	HAF	10,240	<10
	SF	<10	<10
	Sonically vibrated EP–RC	20,480	160
	HAF	20,480	<10
	SF	<10	160

Sonic vibration was carried out for 1 hr.
HAF : Haemagglutinin fraction.
SF : S fraction.

64 to 128. On separation of the HA and S fractions, nearly all S activity was found in the latter and the HA was free of detectable S antigen.

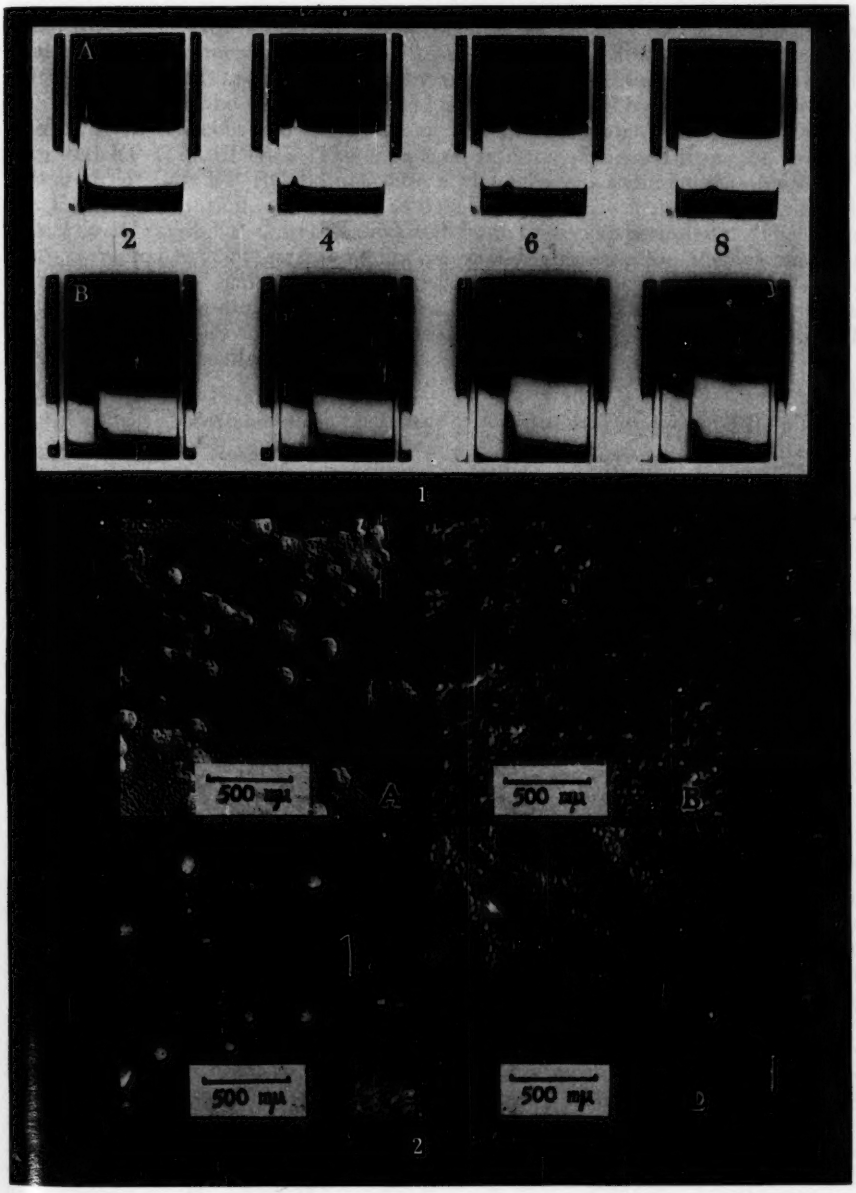
Ultracentrifuge analyses and electron microscopy

The sedimentation velocity constant for several preparations of each of EP–C, EP–RC, the sonically vibrated EP–C and EP–RC, and of the HA and S fractions separated from them was determined, using the Spinco ultracentrifuge model E. The typical sedimentation patterns of EP–C and of EP–C which had been exposed to sonic vibration for 1 hr. are chosen and presented in Fig. 1. As is clearly

EXPLANATION OF PLATE

FIG. 1.—Changes in the ultracentrifuge pattern of purified PR8 virus suspension following sonic vibration. The virus preparation was EP–C (20,480 HA/ml.). A is the pattern of EP–C. B is the pattern after sonic vibration (1 hr.). The direction of sedimentation is from left to right. The numbers in the photograph indicate the time in minutes after the rotor reached 16,200 r.p.m.

FIG. 2.—Electron micrographs of PR8 virus, sonically vibrated virus, and haemagglutinin and S fractions separated from sonically vibrated virus.
A : Elementary particles of PR8 virus (EP–C).
B : EP–C sonically vibrated for 1 hr.
C : Haemagglutinin fraction separated from sonically vibrated EP–C.
D : S fraction separated from sonically vibrated EP–C.



Kato and Okada.

seen, following sonic vibration the sedimentation pattern of EP–C disappeared and a pattern sedimenting at a much lower velocity appeared. The sedimentation patterns of EP–RC and of the sonically vibrated EP–RC were quite similar. The HA purified from the sonically vibrated EP–C or EP–RC by adsorption onto and elution from red cells showed the same sedimentation pattern as the sonically vibrated EP–C or EP–RC. The S antigen fraction separated from the sonically vibrated EP–C or EP–RC did not show a homogeneous sedimentation pattern.

The sedimentation coefficients of EP–C and of the HA purified from the sonically vibrated EP–C were calculated from typical experiments. The values were corrected by plotting the reciprocal of the sedimentation coefficients found for 3 preparations against the concentration at which they were run and extrapolation to infinite dilution. The results are shown in Tables V and VI. It can be seen in the Tables that the sedimentation coefficient of the HA was about 1/10 of that of the elementary particles.

TABLE V.—*Sedimentation Coefficient of Elementary Particles of PR8 Virus*

Experiment	Virus preparation	Virus concentration HA/ml.	Calculated $S_{20,w}$ Svedberg units
1	EP–C	40,960	672
2	EP–C	Diluted 1 : 2	698
3	EP–C	Diluted 1 : 4	730
Value obtained by extrapolation to infinite dilution			752

TABLE VII.—*Sedimentation Coefficient of Haemagglutinin Particles Obtained from Sonically Vibrated PR8 Virus Suspension (EP–C)*

Experiment	Haemagglutination concentration HA/ml.	Calculated $S_{20,w}$ Svedberg units
1	40,960	69
2	Diluted 1 : 2	71
3	Diluted 1 : 4	73
Value obtained by extrapolation to infinite dilution		74

The morphology of EP–C, EP–RC, the sonically vibrated EP–C and EP–RC, HA and S fractions obtained from the sonically vibrated elementary particle suspensions were examined by electron microscopy. Fig. 2A is an electron micrograph of EP–C. It shows spherical particles of 100 to 110 mμ diameter. The electron micrograph of EP–RC showed identical particles. Ten preparations of the sonically vibrated EP–C and EP–RC were examined. Fig. 2B is a typical electron micrograph of EP–C sonically vibrated for 1 hr. It shows numerous smaller spherical particles than the elementary particles, and the intact elementary particles cannot be seen. The electron micrographs of the other sonically vibrated preparations not presented here showed similar features. The HA fraction separated from the sonically vibrated EP–C (Fig. 2C) shows spherical particles of 40–60 mμ diameter. The S fraction (Fig. 2D) shows much smaller spherical particles than the HA particles. The particles formed beaded thread-like aggregates. Therefore, the precise determination of the size of the individual particle is difficult, but the approximate particle size is 15–30 mμ. Visible elements other than these particles are not found in the S fraction. Following high-

TABLE VII.—*The Toxicity of Haemagglutinin and S Fractions of Influenza Virus*

Experiment	Material injected	HA titre/ml.	Dilution	Results of intravenous injection (death rate)
1	Untreated EP–RC	5,120	Undiluted	3/3
			Diluted 1 : 2	3/3
			,, 1 : 4	2/3
			,, 1 : 8	1/3
			,, 1 : 16	0/3
	HA fraction	5,120	Undiluted	3/3
			Diluted 1 : 2	3/3
			,, 1 : 4	1/3
			,, 1 : 8	0/3
			,, 1 : 16	0/3
	S fraction	<2	Undiluted	0/3
			Diluted 1 : 2	0/3
			,, 1 : 4	0/3
2	EP–RC treated with sonic vibration	5,120	Undiluted	3/3
			Diluted 1 : 2	3/3
			,, 1 : 4	3/3
			,, 1 : 8	2/3
			,, 1 : 16	0/3
	HA fraction	5,120	Undiluted	3/3
			Diluted 1 : 2	3/3
			,, 1 : 4	2/3
			,, 1 : 8	0/3
			,, 1 : 16	0/3
	S fraction	<2	Undiluted	3/3
			Diluted 1 : 2	1/3
			,, 1 : 4	0/3
			,, 1 : 8	0/3
			,, 1 : 16	0/3
3	EP–RC treated with sonic vibration	10,240	Undiluted	4/4
			Diluted 1 : 2	4/4
			,, 1 : 4	4/4
			,, 1 : 8	2/4
			,, 1 : 16	0/4
	HA fraction	10,240	Undiluted	4/4
			Diluted 1 : 2	4/4
			,, 1 : 4	2/4
			,, 1 : 8	0/4
			,, 1 : 16	0/4
	S fraction	<2	Undiluted	3/4
			Diluted 1 : 2	1/4
			,, 1 : 4	0/4
			,, 1 : 8	0/4
			,, 1 : 16	0/4

Sonic vibration was performed for 1 hr.

The LD_{50} for mice of each preparation is calculated in terms of the HA titre of the original EP–RC suspension. The values per 10 g. of body weight are as follows:

Exp. 1	Untreated EP–RC	226 HA units
	HA fraction	381 HA units
	S fraction	>1280 HA units
Exp. 2	EP–RC treated with sonic vibration	152 HA units
	HA fraction	304 HA units
	S fraction	765 HA units
Exp. 3	EP–RC treated with sonic vibration	160 HA units
	HA fraction	320 HA units
	S fraction	905 HA units

speed centrifugation of the S fraction at 100,000 *g* for 30 min. the supernatant fluid contained neither the particles nor the S antigen activity. Thus, the particles could be considered as S antigen particles.

Distribution of the toxic agent in the HA and S fractions separated from the sonically disrupted virus particles

The toxicity of both the HA and S fractions separated by the red cell procedure from sonically vibrated virus suspensions was examined. Typical experiments are shown in Table VII. In these experiments EP–RC suspension containing 5,120 HA units per ml. was the original material. This suspension was divided into 3 aliquots, of which 2 were treated with sonic vibration for 1 hr. independently. As shown in the Table, in the case of the untreated EP–RC suspension the toxicity could not be separated from the virus particles by adsorption onto and elution from red cells, although some loss of activity occurred. On the other hand, in the case of the sonically vibrated EP–RC suspensions part of the toxicity could be separated from the HA particles. In other experiments using EP–C suspensions similar results were obtained.

Since the reciprocal of the LD_{50} (in HA units of original EP–RC) of each preparation represents the strength of its toxicity, the ratios between the toxicities of sonically vibrated EP–RC suspension, HA and S fractions may be calculated from the data described in Table VII. In Experiment 2 the relationship is 10 : 5 : 2, and in Experiment 3 it is 12 : 6 : 2. From these values it can be estimated that 1/6 to 1/5 of the toxicity was released from the HA particles, 1/2 was associated with the HA particles and about 1/3 was lost during the fractionation procedures. Considering that this loss of toxicity seemed to occur during the incubation of the red cell–HA aggregates at 37° for 90 min. required for elution, 4/5 to 5/6 of the toxicity might have been associated with the HA particles.

The toxicity associated with the HA particles was inseparable from the particles by a further adsorption onto and elution from red cells although some loss of activity was noted during the procedure. However, certain inactivating agents destroyed the toxicity whereas the haemagglutinating activity was unaffected or only slightly decreased. The results are summarized in Table VIII. It is seen that the separation of these activities was shown by heating at 56° for 30 min., treatment with sodium ethylmercurithiosalicylate (Merzonin) 1 : 10,000 and ultraviolet irradiation for 10 min.

In order to define the relation of the toxic agent released into the S fraction to S antigen particles, separation of toxicity from S antigen activity by high-speed centrifugation was attempted. The results of these experiments are shown in Table IX. As can be seen, the toxic agent was not sedimented in 30 min. at 20,000 r.p.m. but was in 60 min. at 40,000 r.p.m. The activity of S antigen showed a similar sedimentation behaviour.

Pathological changes in mice which died from intravenous injections of the various preparations obtained from influenza virus particles

It has been reported that autopsy of mice killed by intravenous and intraabdominal injections of potent suspensions of influenza A virus revealed mottlings of the liver and intestinal haemorrhages (Henle and Henle, 1946). The autopsy findings in ddN strain mice, employed in the present studies, revealed identical

TABLE VIII.—*Effects of Heat, Merzonin and Ultraviolet Irradiation on the Toxicity and Haemagglutinating Activity of the Haemagglutinin Particles*

Treatment	HA titre/ml.	Results of intravenous injection (death rate)
—	2,560	3/3
Heat	1,280	0/3
Ultraviolet irradiation	2,560	0/3
Merzonin	2,560	0/3
—	10,240	3/3
Heat	5,120	0/3
Ultraviolet irradiation	10,240	0/3
Merzonin	10,240	0/3
—	20,480	3/3
Heat	5,120	0/3
Ultraviolet irradiation	20,480	0/3
Merzonin	20,480	0/3

Heat : Heated at 56° for 30 min.
Ultraviolet irradiation : Irradiated at a distance of 30 cm. for 10 min. using a germicidal lamp (Mitsubishi Electric Co.).
Merzonin : Treated with sodium ethylmercurithiosalicylate (Merzonin, Takeda and Co.) 1 : 10,000 at 37° for 4 hr. and at 4° overnight.

TABLE IX.—*Sedimentation of the Toxic Agent and S Antigen by High-speed Centrifugation*

Material injected	S antigen units/ml.	Results of intravenous injection (death rate)
S fraction	160	3/3
20,000 r.p.m., 30 min.—		
Supernate	160	3/3
Sediment	10	0/3
40,000 r.p.m., 60 min.—		
Supernate	<10	0/3
Sediment*	160	2/3

* Twice concentrated.

lesions after intravenous injections of influenza virus PR8 strain. Moreover, after intravenous injections of toxic doses of sonically disrupted virus particles, and HA and S fractions separated from them, the autopsy findings were identical with those in mice injected with the intact virus.

Nor were any differences found between the histological appearances in mice injected with the various preparations. Histologically, generalized damage to the blood capillaries and related changes were found in the liver, spleen, kidneys, intestines, myocardium, lungs and brain. The most conspicuous changes were found in the liver, small intestine and spleen. The histological findings in the liver, intestines and spleen were quite similar to those already described by Heule and Henle (1946). The histological changes in the kidneys, myocardium and brain were nearly identical with those in rabbits intravenously injected with potent suspensions of influenza virus (Kato and Hara, 1961). In the lungs there was slight

hyperaemia in the septa and little haemorrhages into the alveolar spaces in contrast to the changes in rabbit lungs which showed the picture of acute pulmonary congestion and oedema (Kato and Hara, 1961).

DISCUSSION

It has been reported that sonic vibration liberates some internal S antigen from influenza virus particles, and that its release is not immediately accompanied by detectable losses in the infectivity nor a change in the rate of sedimentation of the virus particles by high-speed centrifugation (Lief and Henle, 1956*b*). In their experiments sonic vibration had to be performed for much longer to reduce the infectivity than to release the internal S antigen. These results led to a curious hypothesis that the release of internal S occurred without the disintegration of the virus particles. However, the results described in the present paper seem to warrant strongly the conclusion that sonic vibration disintegrates influenza virus particles (PR8 strain) nearly completely into subunits, the HA and S antigen particles. Thus, the release of internal S antigen following sonic vibration can be reasonably considered to be the result of the disintegration of the virus particles.

These findings are quite comparable with those of Hoyle (1952), Hoyle *et al.* (1953) and Schäfer (1957), namely that the particles of influenza and fowl plague viruses were disintegrated by ether treatment into the HA and S antigen particles. The differences in the particle size and sedimentation coefficient of the subunits obtained by ether treatment and by sonic vibration may be largely due to the differences of the virus strains and the methods. Schäfer (1959) has described how G-antigen, which seemed to be identical with S antigen, isolated after splitting fowl plague virus particles with ether, showed small spheres and elongated elements, the largest elongated elements appearing to be composed of 6 of these spheres. The electron micrograph of our preparation showed that S antigen particles tended to form long beaded thread-like elements which were composed of many more particles. It is likely that internal S antigen consists of such thread-like structures and is packed tightly within the virus particle. Horne, Waterson, Wildy and Fornham (1960) have revealed an inner helical structure in viruses of the myxovirus group.

The disintegration procedure for influenza virus particles using sonic vibration was found to be superior to ether treatment since the toxicity of the virus particles was not lost with sonic vibration. On separation of the HA and S fractions from the sonically disrupted virus particles by the red cell procedure, 1/6 to1/5 of the toxicity of the virus particles was released into the S fraction. Although the proportion of the toxicity released into the S fraction was small, this result has an important significance. By this finding it can be definitely verified that the toxicity of the virus is an activity independent of the infective and haemagglutinating properties.

The toxicity released into the S fraction could not be separated from S antigen particles by means of differential centrifugation. Even after splitting influenza virus particles with ether, S antigen activity was well maintained (Hoyle, 1952; Hoyle, *et al.*, 1953; Schäfer and Zillig, 1954; Lief and Henle, 1956*a*). But this treatment destroyed completely the toxicity of the virus. Thus, the toxic agent is possibly not an integral component of the antigen.

Even after the distintegration of the virus particles the major part of their toxicity was associated with the HA particles. However, the toxicity of the HA particles was readily destroyed by certain physical and chemical agents, and its destruction was not accompanied immediately by significant losses in the haemagglutinating property. In the case of intact virus particles it was already known that upon exposure to certain inactivating agents infectivity was lost at faster rate than toxicity, and that the haemagglutinating property was the most resistant among these 3 attributes (Henle and Henle, 1946, 1947). Therefore, it is most likely that the toxic agent associated with the HA particles is an adherent component and that the HA itself is non-toxic. Considering the difficulty of releasing it even after the disintegration of the virus particles, the toxic agent seems to adhere firmly to the HA particles.

It has been suggested as a hypothesis that the HA particles are situated near the surface of the elementary particle and S antigen constitutes a central core (Schäfer, 1959; Frisch-Niggemeyer, 1959). Based on this schematical structure, it can be postulated that the majority of the toxic agent is located near the surface of the elementary particle with an intimate relation to the HA. The chemical nature of the toxic agent is still unknown. But its lability to certain physical and chemical treatments suggests that it is a kind of protein. Since ether readily depletes its activity, lipid seems to be an integral component of the toxic agent.

SUMMARY

Sonic vibration disintegrated influenza virus particles (PR8 strain) nearly completely into subunits, the haemagglutinin and S antigen particles, without destroying the toxic agent.

Following this disintegration procedure, some of the toxic agent was released from the haemagglutinin particles. The toxic agent released could not be separated from the S antigen particles by means of differential centrifugation, while the agent seemed not to be an integral component of S antigen. A major proportion of the toxic agent remained associated with the haemagglutinin particles even after the disintegration of the virus particles. However, its toxicity was readily inactivated by certain physical and chemical treatments whereas the haemagglutinating activity was not destroyed.

From its distribution in the subunits it could be postulated that the majority of the toxic agent is located near the surface of the virus particle with an intimate relation to the haemagglutinin.

The pathological changes in mice which died from intravenous injections of toxic preparations free of infectivity were grossly and microscopically identical with those in mice which died from injection of infectious virus particles.

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INDUCTION OF STAPHYLOCOCCAL INFECTIONS IN MICE WITH SMALL INOCULA INTRODUCED ON SUTURES*

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THESE studies were undertaken in the hope of evolving a simple experimental model for staphylococcal infection, the aim being to employ a small inoculum and to approximate natural infection of man in type and site of lesion.

Most of the present methods of producing experimental staphylococcal infection involve techniques which are probably not analogous to the pathogenesis of natural infection. The routes used, such as intraperitoneal, intravenous or intramuscular injection are highly artificial for a micro-organism which, in man, typically causes localized, superficial pus formation. Secondly, the doses needed to infect by these routes are very high. Of current investigators, Gorrill (1951, 1958), has used the smallest doses, but even in this method intravenous injection of 10^5–10^6 staphylococci is required to induce the formation of kidney abscesses in mice. These inocula are probably much larger than those responsible for the common infections of man.

A consideration that appeared important to us is the frequent association of staphylococcal infection with either a " foreign body " or local damage of some sort. For example, in acne vulgaris, sebaceous secretions may become inspissated, plug the ducts and often rupture them, with extrusion of sebaceous material into the surrounding tissues. Wound infection often begins about sutures with the formation of stitch abscesses. It seems likely that the staphylococcus is not usually a primary invader and that the provision of favourable local circumstances should be taken into account in the design of an experimental model of infection.

Elek and Conen's experiments (1957) on human volunteers suggested a possible line of approach. They found that the minimum pus-forming dose (PFD) of *Staphylococcus pyogenes* in human skin was reduced from 10^6 to about 10^2 when the cocci were introduced on silk sutures. The method described in the present article is essentially that of Elek except that mice were used instead of human volunteers and various other suture materials were used in addition to silk.

MATERIALS AND METHODS

Mice.—CFW female mice of 15–20 g. weight were used one week after arrival.

Suture materials.—Black braided silk, 4–0 gauge, Ethicon Inc.; white cotton thread, size 40 machine twist, J. and P. Coats; black braided nylon, 4–0 gauge, Ethicon Inc.; black braided silicone silk, 4–0 gauge, American Cyanamid Co.; black human hair; green braided

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Dacron polyester fibre (Mersilene), 3–0 gauge, Ethicon Inc.; catgut, 4–0 gauge, Ethicon Inc.; steel wire, 4–0 gauge, Ethicon Inc.

All the above materials were boiled for 2–3 min. before use.

Cultures of Staphylococcus pyogenes.—One strain of *Staph. pyogenes* (phage type 52/52A/80/81) was isolated from an infected wound in an experimental dog in a veterinary institution where this strain was prevalent among the personnel. The following cultures were obtained from human sources: 8 strains of phage type 80/81 or 52/80/81; 1 strain of phage type 47/53/54/75/77/VA_4; 1 strain of phage type 3A/3B/3C/55/71; 7 nontypable strains; 4 nontypable stock cultures, probably originally from human sources.

Cultures of coagulase negative Staphylococci.—Eighteen strains isolated from various human sources; 1 strain from an air settling plate in a hospital operating room; 1 stock strain of *Staphylococcus albus* from the Microbiology Department, University of Pennsylvania School of Medicine.

Culture media.—Trypticase Soy Broth was obtained from Baltimore Biological Laboratories. Trypticase Soy Agar was made by incorporating 1·5 per cent agar into the broth. These media were used for phage typing and for propagation of phages. They were employed also for the culture of staphylococci used in the present experiments.

Anaesthetic.—Nembutal, 0·1 ml. of 0·7 per cent solution per 10 g. bodyweight was injected intraperitoneally.

Depilatory.—A commercial cosmetic depilatory (Nair) was used.

Method of inducing infection.—Mice were prepared by depilating under nembutal anaesthesia an area approximately 1·5 cm. square over the shoulder blades.

Sutures were prepared by soaking 2-in. lengths for 30 min. at 37° in an appropriate dilution in broth of an overnight broth culture of a staphylococcal strain. The number of staphylococci picked up by a 2-in. silk suture was about 1/1000 of the number of staphylococci per ml. of suspension; the number picked up by a nylon suture was less, usually about 1/10,000 the number per ml.; for example, a silk suture would pick up 10^4 organisms from a suspension containing 10^7 staphylococci per ml.; a nylon suture would pick up 10^3 staphylococci from the same suspension.

After soaking, the sutures were transferred to sterile filter paper in petri dishes and left at 4° overnight.

The mice were again anaesthetized on the following day and sutures inserted subcutaneously with a fine curved cutting needle, taking a bite of 1·0–1·5 cm. Ends of the suture were cut off just clear of the skin and capped with collodion. The sutures were not tied.

Enumeration of staphylococci on sutures.—The number of staphylococci introduced on the sutures was estimated in the following way. Two unused sutures were put into separate tubes of broth and shaken mechanically for 45 min. at room temperature. Two sets of cut-off ends from the sutures put into mice were treated in the same way and viable counts made on the contents of each tube. The difference between the mean count for the entire suture and mean count for the cut-off ends was taken as the dose inserted.

Bacterial counts were very reproducible and appeared to give good assessment of the dose used provided the numbers to be counted were not too small. The method was tested by shaking sutures repeatedly in fresh tubes of broth and finally cultivating the sutures themselves by stretching them out on agar plates.

Table I gives the results of a typical experiment in which counts were made of staphylococci on sutures. The numbers 1, 2, 3, 4 represent 4 sutures each of which was shaken 3 times. The " working count ", derived from the results of the first shaking, is the count given in most of the following experiments in which the sutures were shaken only once. The total count for each suture is calculated from the total number of organisms recovered from 3 successive shakings. All sutures were laid on agar after the 3rd shaking and yielded only a few scattered colonies on incubation. In other words, 3 successive shakings washed most of the staphylococci from the sutures and permitted reasonably accurate enumeration. The results also show that the working count was a fairly good estimate of the dose used.

RESULTS

Silk sutures

The development of the gross lesions is shown in Figs. 1–4. The first visible sign of infection was thickening of the tissues over the suture so that it was no

TABLE I.—*Recovery of Staphylococci from Silk Sutures upon Repeated Shaking in Broth*

	Number of staphylococci per 0·05 ml. broth* Suture			
	1	2	3	4
First shaking	114	106	159	143
Second shaking	65	67	37	35
Third shaking	26	40	28	28
Total number of colonies . .	205	213	224	207
Total count per suture . . .	$6·2 \times 10^3$	$6·4 \times 10^3$	$6·7 \times 10^3$	$6·2 \times 10^3$
Working count (from 1st shaking) .	$3·4 \times 10^3$	$3·2 \times 10^3$	$4·8 \times 10^3$	$4·3 \times 10^3$

Culture: *Staph. pyogenes*, phage type 52/80/81. Sutures were soaked in a suspension containing approximately 10^6 organisms per ml.
Suture: 4–0 silk.

* Sutures were shaken in 1·5 ml. vols. of broth, of which 0·05 ml. was used for viable counts.

longer visible through the skin. This change was apparent 24–48 hr. after insertion of the suture. Infection progressed fairly rapidly and by the 3rd or 4th day there was usually an obvious abscess. Most of these abscesses began to subside by the 5th or 6th day, particularly if they were draining freely. Abscesses which did not drain early increased slowly in size for as long as 2–3 weeks before subsiding. Recovery was always complete by 5 weeks.

The mice were usually killed on the 5th or 6th day. The local lesion was cultured and pus was looked for along the suture. Pus was sometimes hard to find even with quite a large swelling and might be plentiful when the superficial swelling was slight.

Several groups of mice were used for a rough estimate of the dissemination of staphylococci in the body in this type of infection. Spleen and kidneys were removed aseptically and cut into small pieces with a sterile scalpel. Both kidneys from an animal were minced together. A loopful of each type of tissue suspension was then spread on segments of a trypticase soy agar plate and about 0·05 ml. of heart blood was streaked on another segment of the same plate. All cultures of staphylococci recovered from these plates were phage typed.

In this way 370 mice in all were examined between the 5th and 8th day after infection.

EXPLANATION OF PLATES

FIG. 1.—Appearance immediately after insertion of silk suture. Suture is visible through the skin along its entire length. If no infection takes place it remains visible as long as retained.

FIG. 2.—Infection at 2 days. Thickening of skin obscures the suture. This change is sometimes apparent 24 hr. after insertion of the suture.

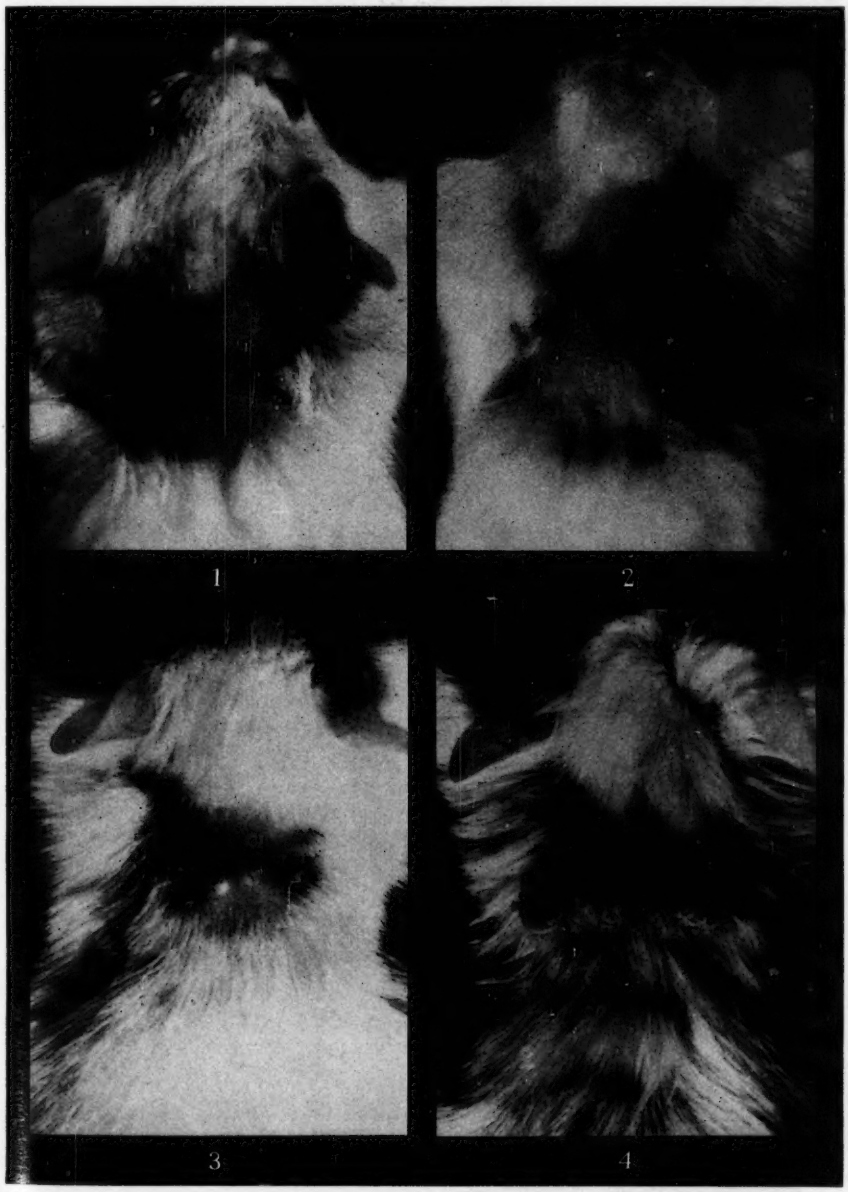
FIG. 3 and 4.—Infection at 4 days. There is usually obvious pus formation.

FIG. 5.—Silk suture carrying *Staph. pyogenes*; 24 hr. after insertion. There is a marked polymorphonuclear and fibrin exudate immediately surrounding the suture with oedema of skin and subcutaneous tissues. ×35.

FIG. 6.—Silk suture carrying *Staph. pyogenes*; 3 days after insertion. Necrosis has occurred with extensive oedema and infiltration of surrounding tissues. ×35.

FIG. 7.—Silk suture carrying *Staph. pyogenes*; 5 days after insertion. Polymorphonuclear infiltration and oedema are extensive in underlying muscle. ×35.

FIG. 8.—Silk suture carrying *Staph. pyogenes*; 5 days after insertion. Polymorphonuclear infiltration of muscle is shown at higher magnification. ×103.



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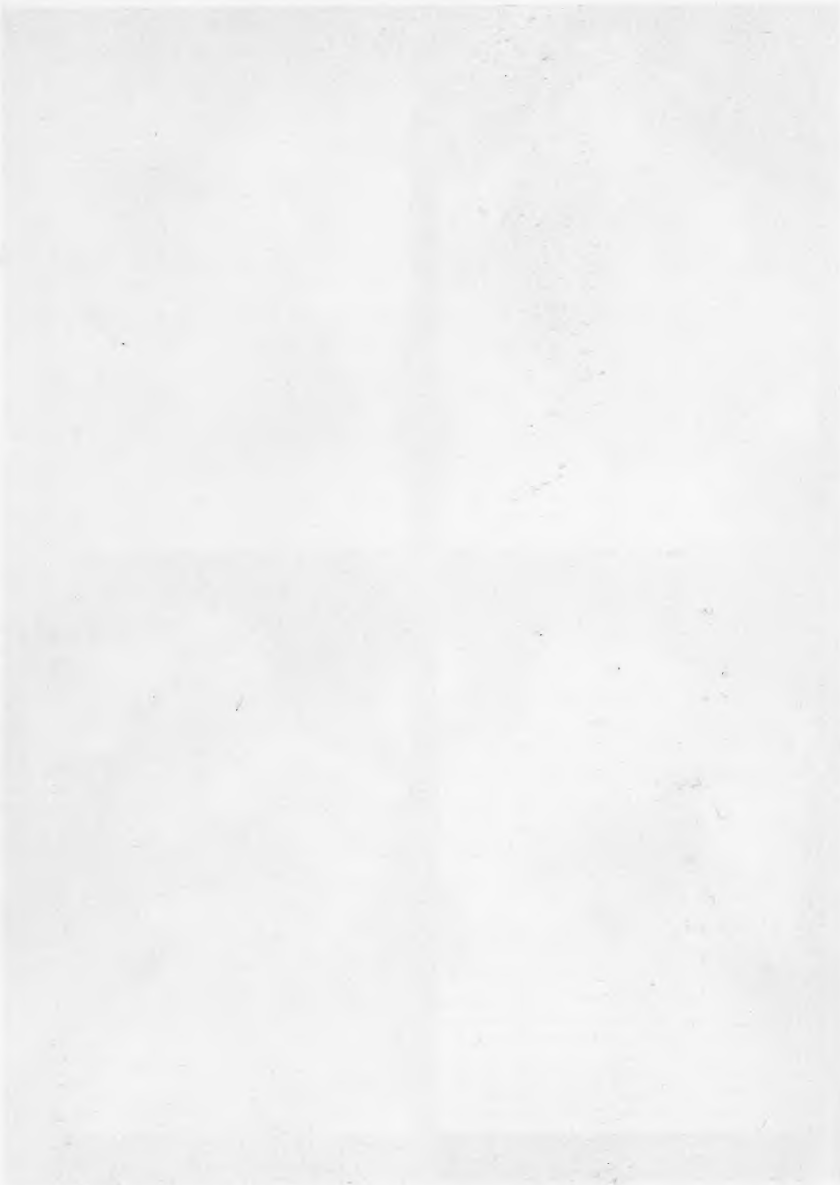

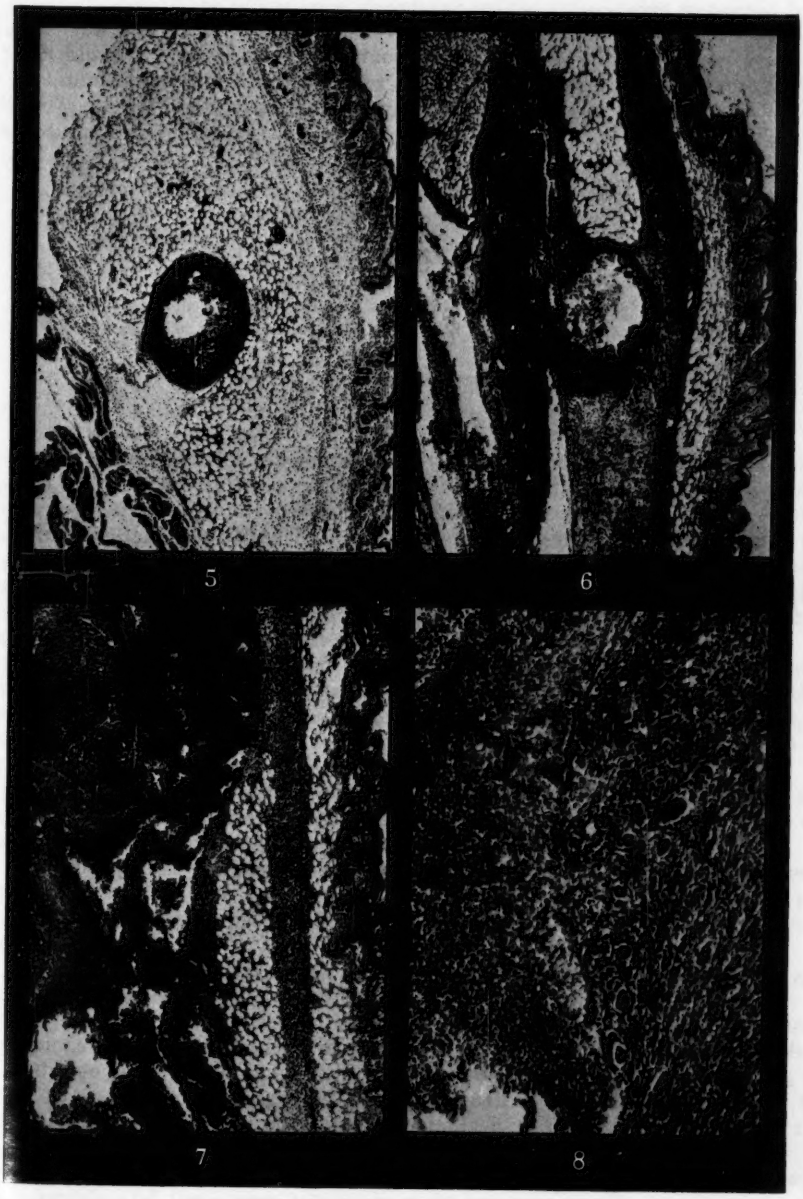

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Staphylococci of the phage type introduced on the suture were recovered from 16 spleens (4·3 per cent) ; 30 specimens of kidney tissue (8 per cent) ; 55 specimens of heart blood (15 per cent). One kidney abscess was found.

Preliminary experiments suggested that the smallest dose which uniformly induced pus formation was 1000–1500 coagulase-positive staphylococci. This dose was used for most of the early work.

TABLE II.—*Production of Abscesses by* 8 *Strains of Staphylococci of Phage Types* 80/81 *and* 52/80/81 *on Silk Sutures*

	Number of staphylococci per suture					
	10^6	10^5	10^4	10^3	10^2	10^1
Number of mice positive*	20	23	21	109	83	31
Number of mice negative	3	1	1	4	35	13
Per cent positive	87	96	95	96	71	70

* Positive = abscess with visible pus present at autopsy ; culture of pus yielded staphylococci.
Suture : 4–0 silk.

Table II summarizes the results of early experiments with 8 strains of phage type 80/81 or 52/80/81 from actively infected patients. The results confirmed the impression that 1000 organisms was the smallest uniformly effective dose. The data also show that the PFD_{50} (the dose resulting in pus formation in 50 per cent of the animals) was well below 100 organisms. If any difference in strain virulence were to be demonstrated by this method employing silk sutures, it would be necessary to use very small inocula of staphylococci, probably smaller than can be counted with reasonable accuracy by our method.

There is a further reservation that concerns the use of very small inocula. Sutures were not always retained subcutaneously but were sometimes lost within the first two days after insertion. When this happened infection might or might not follow. With small inocula relatively more sutures were lost and only 20–30 per cent of these sutureless mice developed infections. If the inoculum was 10^3 or higher, even when the suture was lost, infection followed in 50 per cent of the mice. Mice from which sutures were lost and which did not develop abscesses are not included in the totals shown in the various tables.

The LD_{50} by the intraperitoneal route for the strains used in the experiments summarized in Table II was 5×10^8 cocci, and the dose needed to cause pus formation by subcutaneous or intradermal injection was about 10^7 in the absence of a suture.

A limited survey of other coagulase-positive staphylococci introduced on silk sutures gave similar results, which are recorded in Table III.

The numbers of mice used to test individual strains were too small to draw conclusions about their relative virulence, but the PFD_{50} for the group as a whole was between 10 and 100 organisms. This was slightly higher than the PFD_{50} for the 80/81 strains, which appeared to be less than 10 organisms. However, the counting error at these low doses is unavoidably high and the difference may not be significant.

The same difficulty was encountered with lost sutures ; more were lost with small inocula and there were relatively more negative results among mice with missing sutures.

TABLE III.—*Production of Abscesses by* 14 *Strains of* Staph. pyogenes *other than Phage Types* 80/81 *or* 52/80/81

	Number of staphylococci per suture						
	10^6	10^5	10^4	10^3	10^2	10^1	<10
Number of mice positive*	31	66	68	201	103	36	3
Number of mice negative	3	1	2	16	76	33	19
Per cent positive	91	98	97	92	58	52	14

* Positive = abscess with visible pus present at autopsy; culture of pus yielded staphylococci.
Suture: 4–0 silk.

In each experiment, 3–4 control mice were included and given 10^3 staphylococci of strain 5848 on a suture. This was a strain of phage type 52/80/81 which was originally isolated from a paronychia and had been used extensively in earlier experiments. All these mice, about 70 in number, developed abscesses, but they are not included in any of the totals because cultures were not taken from them.

Coagulase negative staphylococci.—Twenty coagulase negative strains were tested. These gave less consistent results than did the coagulase positive strains, not only because there appeared to be greater differences between strains, but also because single strains behaved differently at different times. It was much more difficult to predict the number of organisms a suture would pick up, but usually the counts were lower than those from similar initial concentrations of coagulase positive staphylococci.

Lost sutures were particularly troublesome when coagulase negative strains were tested in mice. However, 300 mice retained silk sutures bearing inocula ranging from 10^1–10^5 cocci. The results are summarized in Table IV.

TABLE IV.—*Failure of Coagulase Negative Staphylococci to Induce Abscess Formation*

	Number of staphylococci per suture				
	10^5	10^4	10^3	10^2	10^1
Number of mice positive	0	0	0	0	0
Number of mice "questionable"*	0	7	12	9	5
Number of mice negative	4	47	76	102	38

Cultures: 20 strains of coagulase negative staphylococci.
Suture: 4–0 silk.

* Mice recorded as "questionable" developed narrow tunnels of pus or necrotic material along the suture with no visible reaction in the surrounding tissue. Most of these were sterile on culture and staphylococci were recovered from the remainder, usually in small numbers.

None of the mice developed abscesses, but about one in eight was recorded as "questionable". These questionable positives developed narrow ""tunnels" of pus or necrotic material along the line of the suture, with no visible reaction in the surrounding tissues. About half of the questionable positives were sterile on culture and staphylococci were isolated from the remainder, usually in small numbers. *Staph. pyogenes*, strain 5848 was again used as control; 25 mice given 10^3 cocci of this strain all developed abscesses.

Histological reaction to sterile and infected silk sutures

The reaction to sterile silk 24 hr. after insertion was almost as extensive as the reaction to silk bearing coagulase positive staphylococci. There was a heavy polymorphonuclear cell infiltration and fibrin exudate immediately surrounding the suture, with oedema of the skin and subcutaneous tissues. The reaction to sterile silk remained relatively constant for about 2 days and then subsided.

The reaction to infected silk proceeded rapidly to central necrosis by the second or third day with great oedema of the surrounding tissues, and by the 5th day polymorphonuclear cell infiltration extended deep into the underlying muscle (Figs. 5–8).

Reinfection of mice recovered from original infection.—Twenty-six mice infected with 10^3 cocci of *Staph. pyogenes* strain 5848 on silk sutures were kept for 4 weeks, by which time all lesions had healed completely. Seventeen of these mice were then given the same dose of staphylococcus 5848 on silk sutures and the remainder received 10^3 organisms of a staphylococcus of Group II (phage type 3A/3B/3C/55/71).

All these mice developed large abscesses which appeared at about the same time as in previously uninfected mice and were comparable in size. When killed on the 6th day after infection they were found to have very much enlarged spleens with average weight of 350 mg., the largest weighing 700 mg. Spleens from 10 normal animals of the same age averaged 105 mg.

Cultures of the heart blood, the spleen and kidneys were made on all 26 animals as described in an earlier section of this paper. Heavy growths of staphylococci were recovered from 3 spleens, 8 specimens of kidney tissue, and 8 samples of heart blood.

Four colonies from each culture were phage typed. Mice in which second infections were caused by the Group II strain yielded colonies of the Group II type only. All cultures from mice given a second infection with staphylococcus 5848 were of the same phage type (52/80/81).

Cotton sutures

Cotton was used in one experiment only. It resembled silk in its ability to pick up staphylococci from broth, and in its capacity to initiate staphylococcal infection. Histological examinations were not made. It has been reported by Dettinger and Bowers (1957) that tissue reactions to silk and to cotton sutures resemble each other closely in type and extent.

Nylon sutures

Nylon sutures were used in several experiments. They proved much more difficult to handle than silk and one-fourth of the sutures were lost within 2 days of insertion. Counts of staphylococci were also much less reproducible and in general lower than those for silk, the highest obtained being 5×10^5 per suture. Early experiments suggested that nylon is very different from silk as a vehicle of infection and its use was continued in an attempt to establish this point. The results of these experiments are given in Table V.

There were a few questionable positives of the type seen with coagulase negative strains on silk, but most of the results were clear-cut negative or positive. The

TABLE V.—*Production of Abscesses by* Staph. pyogenes, *Phage Type* 52/80/81, *Strain* 5848, *on Nylon Sutures*

	Number of staphylococci per suture				
	10^5	10^4	10^3	10^2	10^1
Number of mice positive*	34	7	3	5	0
Number of mice negative	4	6	25	60	3
Per cent positive	90	55	11	8	—

Suture : 4–0 nylon.

* Positive = abscess with visible pus found at autopsy ; culture of pus yielded staphylococci.

PFD_{50} for staphylococcus 5848 on nylon appeared to be about 10^4, although the number of mice receiving this dose was unfortunately small (Table V).

The PFD_{50} for the same strain on silk was known to be less than 100 organisms. An attempt therefore was made to define the PFD_{50} for silk more exactly. The results are given in Table VI and show that the PFD_{50} is well below 100 organisms, but cannot be measured by our method because of the inaccuracy inherent in the enumeration of small numbers of bacteria.

The data in Tables V and VI do not permit detailed comparison of the infectivity of staphylococcus 5848 on nylon and on silk, but it seems probable that the PFD_{50} on nylon is at least 1000 times greater than the PFD_{50} on silk.

TABLE VI.—*Attempt to Determine the* PFD_{50} *of* Staph. pyogenes, *Phage Type* 52/80/81, *Strain* 5848, *on Silk Sutures*

	Number of staphylococci per suture			
	6×10^2	$9{\cdot}3 \times 10^1$	$3{\cdot}6 \times 10^1$	$2{\cdot}1 \times 10^1$
Number of mice positive*	20	9	11	10
Number of mice negative	0	5	4	4
Per cent positive	100	64	73	71

Suture : 4–0 silk.

* Positive = abscess, with visible pus found at autopsy ; culture of pus yielded staphylococci.

Histological reaction to sterile and infected nylon.—The tissue reaction to sterile nylon was considerably less marked than the reaction to sterile silk. At 24 hr. there was a fibrin reaction around the suture and moderate polymorphonuclear infiltration of the surrounding tissues. Slight inflammation was still present at 3 days but had almost completely subsided at 5 days, by which time there was a proliferation of fibroblasts and a few macrophages had appeared.

Nylon carrying small doses of coagulase positive staphylococci induced a more extensive reaction than sterile nylon and it took longer to subside. The reaction was still quite marked at 5 days and was surrounded by a wide zone of fibroblasts. The reaction to heavily infected nylon was indistinguishable from the reaction to infected silk.

Silicone-treated silk

It seemed possible that the superiority of silk and cotton over nylon as vehicles of infection was related to the wettability of the suture material. For this reason

silicone-treated silk was used. This material is said to be non-wettable, but retains the handling properties of untreated silk.

Silicone-treated silk was used in two experiments with staphylococcus 5848 and proved to be very similar to untreated silk in its ability to enhance infection. It also resembled silk in the number of organisms picked up from a given suspension of staphylococci and in yielding reproducible bacterial counts.

The results of experiments employing silicone-treated silk are recorded in Table VII.

TABLE VII.—*Production of Abscesses by* Staph. pyogenes, *Phage Type* 52/80/81, *Strain* 5848, *on Silicone-treated Silk*

	Number of staphylococci per suture			
	10^3	10^2	10^1	<10
Number of mice positive*	21	32	10	7
Number of mice negative	0	1	4	8
Per cent positive	100	97	71	47

Suture : 4–0 " Silicone Silk ".
* Positive = abscess, with visible pus found at autopsy ; culture of pus yielded staphylococci.

Histological reaction to sterile silicone-treated silk.—The tissue reaction to sterile silicone silk was much more extensive than the reaction to any of the other materials used. A heavy fibrin exudate formed around the suture and extensive polymorphonuclear infiltration of the surrounding tissues occurred. There was still a considerable amount of inflammation at 5 days but at this stage there were many macrophages and a fairly marked fibroblastic reaction.

Human hair

Staphylococcal infection is frequently associated with hair follicles, and it is also said that barbers are prone to develop purulent infections around fragments of hair which become imbedded in the skin of their hands, especially in the finger webs. These observations suggested that hair might be an effective agent for introducing staphylococci. Preliminary tests showed that staphylococci did not stick well to human hair. It was necessary to use concentrated suspensions to achieve inocula of more than 10^4 organisms. Doses from 10^2–10^5 cocci were finally introduced with results as shown in Table VIII.

TABLE VIII.—*Production of Abscesses by* Staph. pyogenes, *Phage Type* 52/80/81, *Strain* 5848, *on Human Hair*

	Number of staphylococci per suture			
	10^5	10^4	10^3	10^2
Number of mice positive*	2	1	0	0
Number of mice negative	18	8	9	8
Per cent positive	10	11	—	—

* Positive = abscess, with visible pus found at autopsy ; culture of pus yielded staphylococci.

From the results with human hair shown in Table VIII it can be seen that relatively high doses were needed to initiate infection and that hair was the least effective of the suture materials tried. However, hair appears to have a mild potentiating effect on staphylococcal infection in the mouse skin.

Histological reaction to sterile human hair.—The tissue reaction to sterile human hair was quite marked at 24 hr. There was a fairly heavy polymorphonuclear reaction and a fibrinous exudate around the hair, but by 3 days inflammation had almost completely subsided and at this stage it was much less marked than the reaction to any of the other materials used.

Dacron (Mersilene)

All the above suture materials appeared to enhance infection in rough proportion to the ability of the sterile material to induce an inflammatory response in the tissues. Dacron has been advocated as a suture material largely because the tissue reaction to it is said to be slight. It was used to test the suggested relationship between tissue reaction and infection. One experiment only was done, using staphylococcus 5848 and "Mersilene" suture material. The results are given in Table IX.

TABLE IX.—*Production of Abscesses by* Staph. pyogenes, *Phage Type* 52/80/81, *Strain* 5848, *on Dacron Sutures*

	Number of staphylococci per suture		
	$1{\cdot}4 \times 10^3$	$2{\cdot}1 \times 10^2$	<10
Number of mice positive* .	8	9	4
Number of mice negative .	0	0	3

Suture: 3–0 "Mersilene".
* Positive = abscess with visible pus found at autopsy; culture of pus yielded staphylococci.

This experiment is not strictly comparable to those where silk, nylon, and silicone silk were used, because at the time of the experiment the "Mersilene" suture was available only in 3–0 gauge which is slightly thicker than the other suture materials used. However, as can be seen from the results shown in Table IX, it seems likely that in its ability to enhance infection, dacron resembles silk and silicone-treated silk rather than nylon. Silk and dacron also resembled each other in their ability to pick up similar numbers of coagulase positive staphylococci from a suspension.

Histological reaction to sterile dacron sutures.—At 24 hr. the tissue reaction to sterile "Mersilene" was for the most part very similar to the reaction to nylon, but a few sections showed a more extensive reaction which almost equalled the effect of silicone-treated silk. Proliferation of fibroblasts and invasion by macrophages occurred earlier than with any of the other materials and by the 5th day these cells had largely replaced the acute inflammatory exudate.

Catgut

Catgut was used on one occasion only because the bacterial counts were very low and too variable to permit reasonable estimate of the dose used.

Steel wire

Steel wire picked up staphylococci very well and gave the most reproducible counts of any material used. Repeated shaking also showed that over 90 per cent of the organisms were removed at the first shaking and these counts were therefore probably more accurate than with any of the other materials. However, steel wire was found to be very difficult to handle. Thirty-five out of 40 mice lost their sutures within 24 hr. and for this reason its use was not continued.

DISCUSSION

The observations described demonstrate that subcutaneous abscesses can be produced consistently in mice by small numbers of coagulase positive staphylococci if the bacteria are introduced on certain suture materials, such as silk, silicone-treated silk, cotton, and dacron. All of these materials appear to be roughly comparable in their ability to potentiate infection by small numbers of staphylococci. With other suture materials such as nylon thread and human hair, much larger inocula of staphylococci were required. The potentiating effect of catgut and steel wire could not be tested adequately because of technical difficulties.

Among the 22 strains of coagulase positive staphylococci that were tested, it was not possible to distinguish differences in virulence, although with further experience this might be feasible.

Coagulase negative strains inserted on sutures uniformly failed to cause abscess formation. Great variability was found among coagulase negative strains and even among different cultures of the same strain, in their ability to be picked up and retained by the suture materials.

The effectiveness of the various suture materials in potentiating abscess formation when small inocula of coagulase positive staphylococci were used appears to depend upon two properties: their ability to pick up and retain staphylococci from broth cultures; and the severity of the polymorphonuclear inflammatory reaction caused by implantation of sterile sutures in mouse skin. For example, silk, silicone-treated silk, cotton and dacron, all highly effective as potentiators, are able to pick up about 10 times as many staphylococci per unit length as nylon. Human hair was even less effective than nylon in picking up the cocci. Furthermore, sterile silk, silicone-treated silk and dacron, all of which are effective potentiators, produce a marked and persistent polymorphonuclear inflammatory reaction in mouse skin, whereas nylon and human hair, which are weak potentiators, cause a much milder inflammatory response.

With the more effective suture materials, introduction of approximately 10^3 coagulase positive staphylococci caused abscess formation in almost all mice. Attempts to define the number of cocci that would induce abscess formation in 50 per cent of the animals were only partially successful because the methods do not provide a good estimate of inocula of less than 100 cocci. The errors involved in recovering small numbers of staphylococci from the sutures are great and they are exaggerated by the difficulties inherent in counting small numbers of bacteria. Sutures carrying small inocula are also more liable to be lost than those carrying larger doses. However, if the suture is retained, abscess formation takes place in a large proportion of animals given an inoculum of 10–100 staphylococci.

After introduction of contaminated silk sutures and other materials that act similarly, abscess formation was usually apparent within 4–5 days. At this time cultures of the blood, spleen and kidneys were found to be positive in a small percentage of the animals. If the abscess ruptured, which was common early in the course of the infection, complete recovery occurred promptly. If the abscess did not rupture, healing was much slower, but in most cases was complete in about 4–5 weeks. At this time reinfection at the same site with the same dose of the homologous strain or one of a different phage type, resulted in the development of abscesses which did not differ significantly from those seen at the first infection. Moreover, blood and organ cultures were commonly positive in reinfected mice. It would appear, therefore, that if immunity develops in association with recovery from the primary infection, it is slight in degree, because it provided no demonstrable protection against reinfection under the conditions employed. However, it is possible that immunity might be demonstrable if smaller inocula were used for reinfection.

With respect to immunity it should be noted that staphylococcal infections of the skin in man are characterized by a remittent course with multiple episodes of furunculosis that commonly recur over periods of months to years. Intervals of relative or complete freedom from overt lesions often appear, but there is little evidence that recovery from one bout confers immunity against a second, even though the infecting staphylococci belong to the same phage type on both occasions. If anything, the reverse seems to be the case, because a person who has recovered from an attack seems to be more susceptible than normal to staphylococcal infection.

The method described appears to possess some of the features required of an experimental model of human staphylococcal infections. In particular its simplicity, reproducibility, the use of small inocula and the production of an easily observable, superficial lesion, commend its trial in the investigation of staphylococcal disease. Materials other than those studied might be found which would be more suitable especially for the induction of infections with less than 100 cocci. For example, a suture of some soluble substance such as calcium alginate would permit more accurate estimation of dose.

Although our results show that various suture materials differ greatly in their ability to potentiate staphylococcal infections in young mice, it should not be concluded that these differences pertain also to the use of the same sutures in man. Elek (1957) has demonstrated that silk thread has a striking effect in reducing the inoculum of staphylococci needed to cause abscesses in the skin of human volunteers. The present studies show that silk and certain other materials behave similarly in young mice. On the other hand, preliminary experiments indicate that nylon sutures, which require a relatively large inoculum of staphylococci in young mice, can cause abscesses with much smaller inocula when older mice are used.

It is tempting to draw conclusions from the resemblance of these experimental infections to many human staphylococcal infections. The gross and histological appearances of the lesions, their evolution, and the ease with which mice can be reinfected, all suggest analogies with superficial staphylococcal infection in man. However, it remains to be seen whether any valid comparisons can be made and whether this method can provide information on the course of staphylococcal infection in any animal other than the mouse.

SUMMARY

Local skin abscesses have been induced in young mice employing small inocula of various strains of *Staph. pyogenes* introduced on a number of suture materials.

Differences in virulence among various strains of coagulase positive staphylococci were not apparent.

Coagulase negative strains of staphylococci did not give rise to local abscesses.

Of the sutures used, silk, silicone-treated silk, cotton and dacron were most effective in potentiating infection. Nylon and human hair were less effective in that they required larger inocula of staphylococci.

The potentiating effect of the suture materials appeared to be related to the severity of the polymorphonuclear inflammatory reaction which the sterile materials caused in the mouse skin and to the capacity of the sutures to pick up staphylococci from broth cultures.

Mice that had recovered from one infection showed no evidence of increased resistance upon reinfection with homologous or heterologous strains.

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THE OCCURRENCE OF RENAL CHANGES, RESEMBLING NEPHROSIS, IN RATS POISONED WITH FLUOROCITRATE

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CERTAIN fluoro compounds are incorporated into the citric acid cycle of cells (Gal, Peters and Wakelin, 1956). Fluorocitrate is synthesized which blocks the tricarboxylic acid cycle at the aconitase stage (Peters, Wakelin, Buffa and Thomas, 1953). This constitutes a lethal synthesis, blocks this metabolic pathway and causes an accumulation of citrate (Buffa and Peters, 1949). Among several carbon-fluorine compounds investigated, 1-deoxy-1-fluoroglycerol behaved in an interesting way, because it was toxic only when the liver acted upon it previously (O'Brien and Peters, 1958). There was, as it were, a double lethal synthesis, (1) conversion by the liver tissue to a FC_2 fragment, which was then (2) synthesized to fluorocitrate. This led to the idea that in rats bearing a transplantable hepatoma, this hepatoma might be especially sensitive to 1-deoxy-1-fluoroglycerol, as the hepatoma might be expected to perform a local lethal synthesis. This was tried with negative results. At the same time experiments were made with the recently isolated fluoro-oleic acid (Peters, Hall, Ward and Sheppard, 1960), and with a specimen of synthetic fluorocitrate (Na salt) for which we are indebted to Mr. R. Brown. During these experiments it was noticed that " tumour bearing " rats treated with fluorocitrate showed marked kidney damage resembling the condition in man variously named nephrosis, parenchymatous nephritis or the early stage of type 2 nephritis (Ellis, 1942). The biochemical lesion induced by fluorocitrate is a specific one, and confined to inhibition of the aconitase stage of the Kreb's citric acid cycle. The changes observed led us to investigate the effect of fluorocitrate on the kidney of normal rats. When the right regime and dosage were used fluorocitrate produced fatty degeneration of the renal convoluted tubules and arteries—a very similar histological picture to that found in human nephrosis.

MATERIALS AND METHODS

Rats of both sexes of the August strain (140–200 g.) were used for the transplantation of the hepatoma. This strain of rat was also used to study the effect of fluorocitrate on normal, non-tumour bearing rats. When the tumours were about 2 cm. long the rats were divided into 2 groups by random selection. One group received daily intraperitoneal injections of the fluoro-compound dissolved in 0·9 per cent sterile saline, the other group was injected with equal volumes of saline. The rats were weighed and the tumours measured by calipers and the outlines drawn on graph paper. The rats were killed when ill or when the tumour was large. At autopsy the weight of the tumour was noted and pieces of heart, lungs, liver, spleen and kidneys were taken for histological section. After fixation in formol saline the tissues were dehydrated, embedded in wax, sectioned and stained with haematoxylin and

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eosin. As soon as the renal lesions were observed in the "tumour-bearing" rats treated with fluorocitrate, pieces of kidney were fixed in Baker's solution and frozen sections were stained with Scharlach R for lipid. Picro-Mallory and H. and E. stained sections from wax embedded material were also studied. In 2 experiments normal non-tumour bearing rats were treated with fluorocitrate at various dose levels and their kidneys and viscera compared with those from normal rats injected with saline. Whenever possible heart blood was taken at autopsy for estimation of total serum protein and total blood cholesterol.

Fluorocitrate.—No biosynthetic fluorocitrate was available. The specimen of sodium fluorocitrate used was prepared synthetically by Rivett's method (1953) by Mr. R. Brown, to whom we are indebted. As has been shown (Ward and Peters, 1961), it does not require activation with alkali for experiments *in vivo*; it has approximately 40 per cent of the activity of biosynthetic fluorocitrate.

RESULTS

Renal lesions in non-tumour bearing rats treated with fluorocitrate

It is convenient to describe first the kidney damage occurring in non-tumour bearing rats after treatment with fluorocitrate. Although these were some of the last experiments to be done (Exp. 12 and 13) they threw most light upon the pathogenesis of the renal lesions, especially when the rats were grouped according to the time which elapsed between the injection of fluorocitrate and death.

Renal lesions in rats dying about 2 hr. after fluorocitrate.—Doses of 60 or 80 mg./kg. were usually fatal in about 2 hr. (2 and 8 rats). The kidneys were enlarged and both cortex and medulla were deeply congested. On histological examination Bowman's capsule was distended with exudate to a lesser degree or greater degree (+ to ++++). The cells of the proximal convoluted tubules showed acute degeneration with loss of mitochondria, swelling of cytoplasm and pyknosis of their nuclei. Protein casts, desquamated cells and debris were present in the tubules (Figs 1 and 2). Stainable lipid was absent or no more than a trace was present in tubules, medulla or vessels. The changes observed were consistent with the following sequence of events. First swelling of the cells of the proximal convoluted tubules due to marked swelling of the brush border and a hyaline droplet degeneration of the mitochondria (Fig. 3) in the rest of the cell (Change A). Then progressive loss of the brush border and the cytoplasm of the cell so that it no longer stains red with picro-Mallory (Change B). Finally the death of the cell with pyknosis of the nucleus and loss of most of the cytoplasm (Change C). The proximal convoluted tubules had become acutely blocked by the swollen cells and cellular debris (Fig. 1, 2, 3). Proximal to the block the tubules and Bowman's capsule had become distended with retained glomerular filtrate and debris (Fig. 1 and 2).

Renal lesions in rats dying about 4 hr. after fluorocitrate.—Two rats died after 60 mg./kg., two after 40 mg./kg. and one after 35 mg./kg. In all these rats, dying about 3½ hr. after injection of fluorocitrate, the kidneys were large and deeply congested and the renal surface was irregular like morocco leather. The changes were essentially similar to those of the more acute deaths.

Renal changes in rats recovering from one dose of fluorocitrate and dying, or killed after a second dose.—Two female rats were given a first dose of 40 mg./kg. and after 2 days were given a second dose of 20 mg./kg. One died in 3 hr. 35 min. and the other in 6 hr. 20 min. after the 2nd dose. Two female rats given a first dose of 40 mg./kg. survived a 2nd dose of 15 mg./kg. given 2 days later; and 1 male rat survived after an initial dose of 35 mg./kg. and a 2nd dose of 15 mg./kg.

These animals were killed 2 days after the 2nd dose. The renal changes in these rats were similar in most respects. All the kidneys were larger than those of the control rats. The subcapsular surface was irregular like morocco leather, the cortex was pale, and the medulla pink or red. Frozen sections stained with Scharlach R showed marked fatty degeneration in the convoluted tubules, the cells of which were full of fine droplets of lipid (Fig. 5 and 6). The rats which died showed fatty change in the media of the arteries. These changes were present in the afferent arterioles of the glomeruli (Fig. 5), in the interlobular, and in the arcuate arteries (Fig. 9). Wax embedded sections stained with picro-Mallory showed fuchsinophil material in the media of the arteries (see Fig. 10). The arteries of the rats which were killed showed no stainable lipid, but traces of the fuchsinophil material were seen in the media of the arteries. The rats which died showed more fatty change in the medulla than the rats which were killed, there was also more obvious tubular degeneration in the cortex and more casts in the tubules. Pleural exudate was present and a low serum protein.

EXPLANATION OF PLATES

FIG. 1.—Kidney of non-tumour bearing rat, acute death 2 hr. after fluorocitrate 80 mg./kg. i.p. Three glomeruli show distension of capsular space and distension of first part of proximal convoluted tubule with pyknosis of nuclei. There is swelling of brush borders and cytoplasm of the cells further along the tubule (left upper quarter). Picro-Mallory stain. ×219.

FIG. 2.—Kidney of non-tumour bearing rat, acute death 2 hr. after fluorocitrate 80 mg./kg. i.p. Distension of capsular space and dilatation with necrosis of first part of the proximal convoluted tubule. The middle portions of the proximal convoluted tubules show swollen cells and blocked lumens. Picro-Mallory. ×219.

FIG. 3.—Kidney of non-tumour bearing rat, acute death 2 hr. after fluorocitrate 60 mg./kg. i.p. The tubule below the left glomerulus shows hyaline droplet degeneration of the cells, with blockage of the lumen. There is exudate in the capsular spaces. Picro-Mallory. ×219.

FIG. 4.—Kidney of tumour bearing rat which died 4½ hr. after a second dose of fluorocitrate total dose, 45 mg./kg. The medullary tubules contain hyaline casts, there is engorgement of the capillaries. Picro-Mallory. ×175.

FIG. 5.—Frozen section stained with Scharlach R of kidney of non-tumour bearing rat, which died 6 hr. 20 min. after a second dose of fluorocitrate (total 60 mg./kg. i.p.). Note the arterioles, top left, with lipid droplets in the wall. A number of the tubules show fine lipid droplets near the basement membrane. ×175.

FIG. 6.—Kidney of non-tumour bearing rat, which was killed 48 hr. after second dose of fluorocitrate; 40 mg./kg. given day 0, 15 mg./kg. day 4, killed day 6. Note many lipid droplets in basal parts of tubular cells. Some of the tubules are dilated and the cells are degenerate. Frozen section, Scharlach R. ×175.

FIG. 7.—Kidney of hepatoma-bearing rat killed 24 hr. after 4 doses of fluorocitrate (total 46 mg./kg.). The small artery shows lipid in the muscular coat and all the tubular cells show numerous small lipid droplets. Frozen section, Scharlach R. ×175.

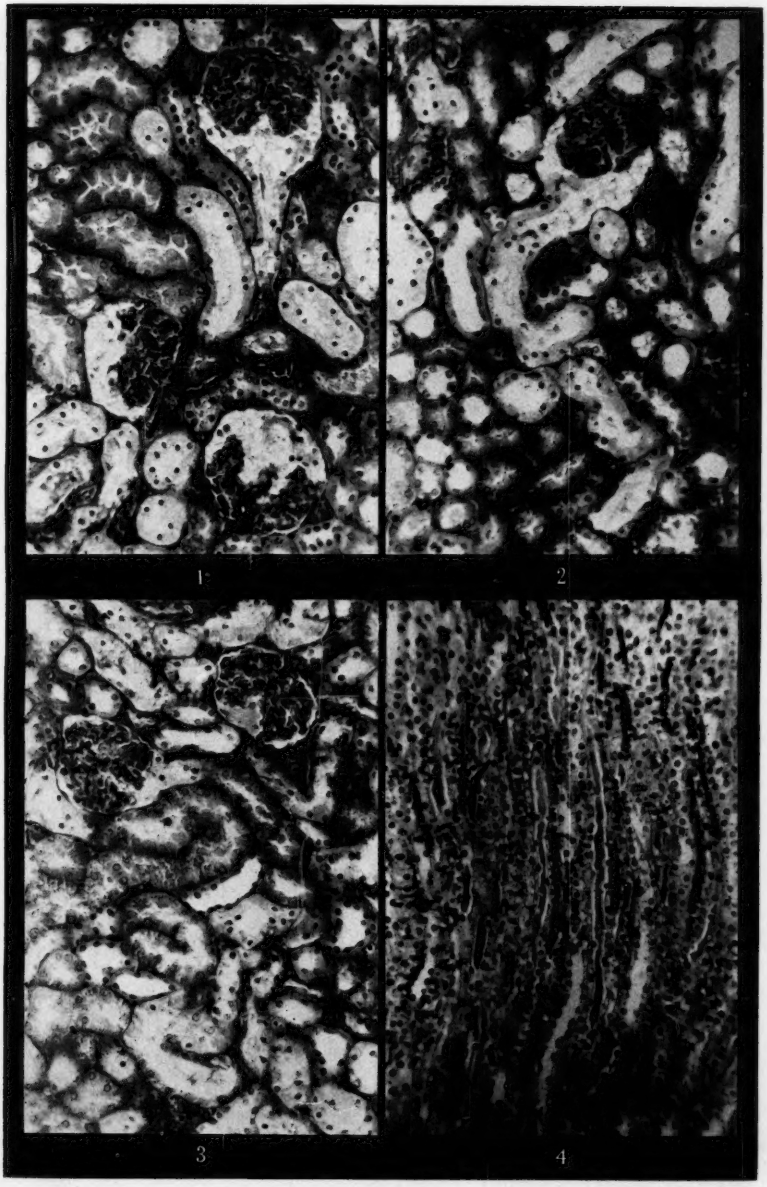
FIG. 8.—Kidney of hepatoma-bearing rat killed 3 days after last of 5 doses of saline. Tubular architecture is normal and there are no droplets of lipid. Frozen section, Scharlach R. ×175.

FIG. 9.—Arcuate artery and interlobular artery of non-tumour bearing rat which died 3½ hr. after a second dose of fluorocitrate (total 60 mg./kg.). Note heavy lipid deposits in muscular wall of artery and its branch. Some lipid is present in walls of tubules but some tubules are full of a granular deposit. Frozen section Scharlach R. ×175.

FIG. 10.—Note fuchsinophil material in media of interlobular artery and branch: wax section stained picro-Mallory from same rat as Fig. 9. The tubules above and below the left glomerulus are full of granular debris. ×245.

FIG. 11.—Extensive tubular degeneration with formation of casts in same kidney as Fig. 4. Hepatoma rat died 4½ hr. after second dose fluorocitrate, total dose 45 mg./kg. Picro-Mallory. ×210.

FIG. 12.—Extensive tubular degeneration and a mass of fibrin in the capsular space in kidney of hepatoma-bearing rat which died 26 hr. after second dose of fluorocitrate (total 25 mg./kg.). Picro-Mallory. ×175.



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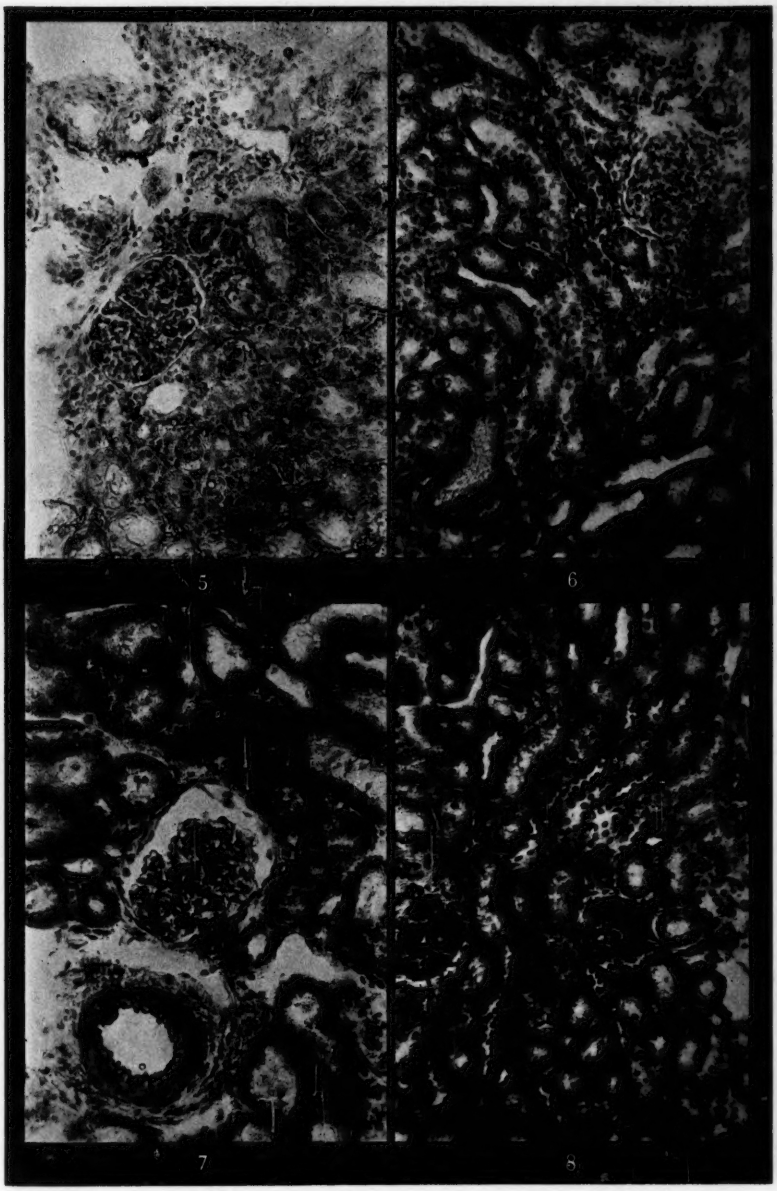

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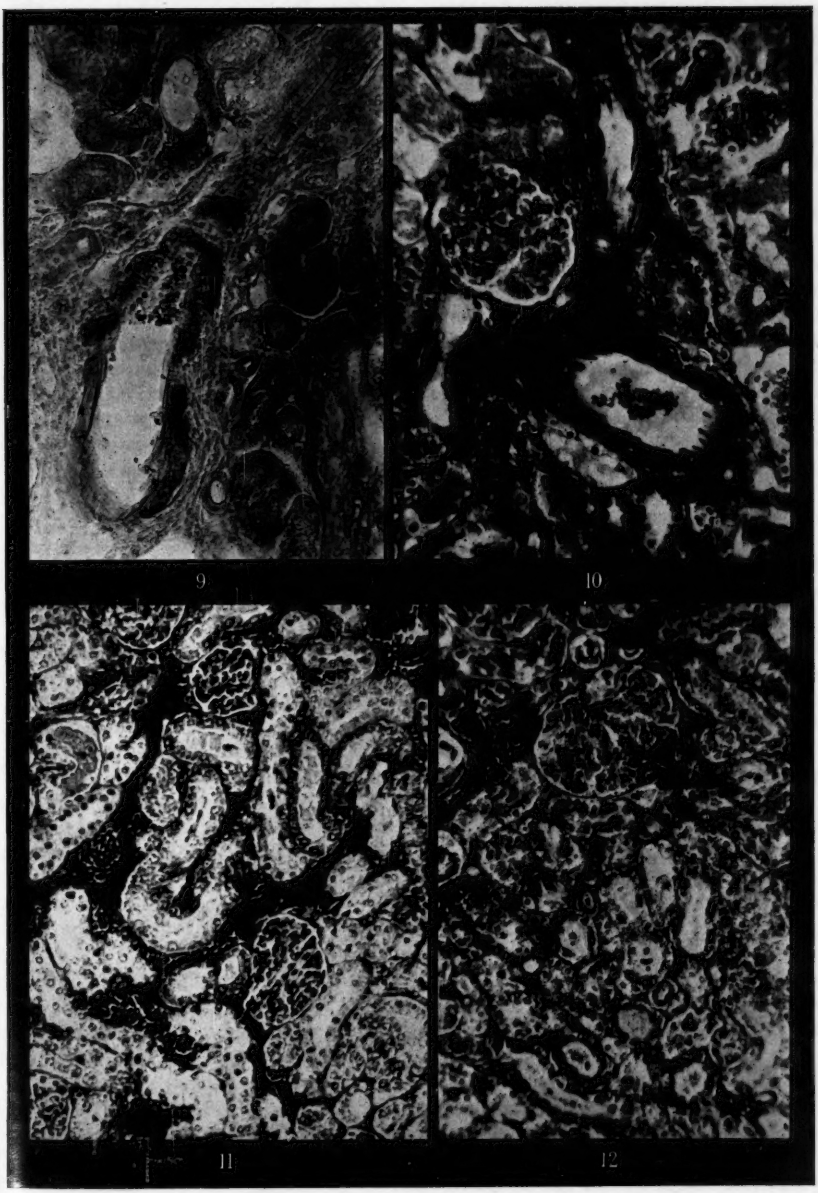

Cater and Peters.

The renal changes seen in these subacute deaths differed from those seen in the acute deaths in certain important respects. There was no distension of Bowman's capsule with exudate. There was the presence of extensive fatty change in tubule and arterial wall. There were signs not only of tubular damage and degeneration but also signs of tubular regeneration. Mitosis was seen in the tubular epithelium of every rat in this group.

Renal changes after multiple doses of fluorocitrate.—A group of 3 rats survived multiple doses of fluorocitrate totalling 150 mg./kg. in 10 days. One was killed 2 days, and two were killed 4 days after the last dose. A fourth rat died on the 9th day of this régime after 132 mg./kg. All the kidneys were enlarged, pale, and with poor demarcation between cortex and medulla. The subcapsular surface was nodular. On histological examination no obvious exudate was seen in Bowman's capsule but one rat showed thickening of the wall of Bowman's capsule and capsular-glomerular adhesions. All kidneys in this group had a moderate amount of fine lipid droplets in the cells of the proximal convoluted tubules, with signs of both tubular degeneration and regeneration, and some round-cell infiltration. Protein casts were present in the tubules. Two rats showed lipid in the medulla, but no lipid was detected in the walls of arteries or arterioles. In this experiment (No. 12) the early doses were small 15 mg./kg. on day 0, 10 mg. day 1, 5 mg. day 2 and 3, 10 mg. day 4, 15 mg. day 7, 60 mg. day 9 and 30 mg. day 10. On the basis of the findings it was decided to do expt. 13 described above in which a big initial dose was given, sufficient to inflict considerable damage to the kidney at the beginning of the experiment. It then became evident that severe initial damage to the kidney made the rats more susceptible to subsequent injections of fluorocitrate.

Control rats.—Eight normal rats (3 males and 5 females) were given intraperitoneal injections of saline equal in number and amount to the injections given to the fluorocitrate treated rats. The kidneys of these rats showed no stainable lipid in tubules or vessels and were essentially normal in every respect. The data on the total serum protein and cholesterol of the normal saline-treated rats, fluorocitrate-treated rats, and tumour-bearing rats treated with saline and treated with fluorocitrate are shown in the Table.

Renal lesions in tumour-bearing rats treated with saline.—Twenty-seven control, hepatoma-bearing rats injected with saline were specifically examined for stainable lipid in the kidney. In 3 rats traces were found in the proximal convoluted tubules, in 3 rats traces were present in the medullary tubules and in 21 no stainable lipid was detected (Fig. 8). In 38 control rats (25 males and 13 females) the kidneys were examined by haematoxylin and eosin and picro-Mallory stained sections. In 22 some protein casts were present in the tubules which were slightly dilated in 6 rats. The presence of the tumour therefore had some deleterious effects upon the kidney by comparison with the 8 non-tumour bearing rats treated with saline injections.

Renal lesions in tumour-bearing rats treated with fluorocitrate.—Twelve rats died 4–26 hr. after a total of 30–45 mg./kg. fluorocitrate given in two doses on successive days. (Another rat died 18 hr. after 3 doses, and one 40 hr. after a 4th dose.) The kidneys of all these rats showed acute necrosis of the cells lining the proximal convoluted tubules with pyknosis or karyolysis of the nuclei (Figs. 11 and 12). Fig. 4 shows the medulla of one of these rats with the tubules full of casts. In some rats fibrosis was present in the capsular space (Fig. 12). The tubules were

TABLE.—*Total Serum Protein g./100 ml.*

	Non-tumour bearing rat injected with		Hepatoma-bearing rat injected with	
	Saline	Fluorocitrate	Saline	Fluorocitrate
	Total Serum Protein g./100 ml.			
	6·7	4·6	6·75	4·8
	6·1	4·8	4·8	5·9
	5·4	6·3	5·45	4·9
	5·7	6·1	4·8	5·8
	7·3	5·6	6·1	5·1
	7·55	7·55	5·2	5·2
	7·8	7·3	5·6	4·65
		7·4		4·7
Mean	6·65	6·206	5·53	5·13
S.E.M.	±0·355	±0·41	±0·267	±0·17
P		0·45	0·03	0·005
	Total Serum Cholesterol mg./100 ml.			
	63	63	63	54
	63	63	84	76
	64	63	125	44
	63	63	106	45
	69	87	63	63
	87	88	113	83
	87		83	83
				65
Mean	70·85	71·15	91·00	64·12
S.E.M.	±4·26	±5·23	±9·20	±5·56
P		0·95	0·05	0·35
			0·03	

full of protein, granular and cellular casts. Any cells which were not completely necrotic showed marked accumulation of stainable lipid near the basement membrane (all +++). Six rats killed 24 hr. after the last of 4 or 5 daily doses (total 37·5 to 57 mg./kg. fluorocitrate) all showed fatty degeneration of the cells of the proximal convoluted tubules (+, ++, ++, +++, +++, +++) but there was tubular dilation with some degeneration of the cells instead of extensive necrosis (Fig. 7). The tubules contained protein, casts and cellular debris. Three of these rats showed stainable lipid in the walls of small arteries in the kidney (Fig. 7), and in 4 there was lipid in medullary tubules. A group of 3 rats was killed 48 hr. after the last of three doses (total 25–28 mg./kg. fluorocitrate). All the kidneys show stainable lipid in the cells of the proximal convoluted tubules (+, + and ++) and in the latter case also in the medullary tubules. The tubules were dilated and casts were present. In 5 rats killed 72 hr. after the last of 5 doses (total 23·5 to 32·5 mg./kg. fluorocitrate) the convoluted tubules were dilated and casts were present, but regeneration was in progress judged by the fact that 3 out of 5 showed mitotic figures. The amount of lipid present was variable (−, −, trace, +, +++). In two of these rats some exudate was present inside Bowman's capsule.

The effect of fluoro-oleic acid treatment on rats with transplantable hepatoma.—Ten hepatoma-bearing rats were given intraperitoneal injections of fluoro-oleic acid, either daily or on alternate days, to a total dosage of 100–150 mg./kg. Their

tumours grew somewhat less vigorously than in the 10 saline-injected controls. However the fluoro-oleic treated rats showed signs of shock, rapid respiration or malaise 2–4 hr. after injection and it was thought that this might account for the difference between the groups. The kidneys did not show any obvious differences between the tumour-bearing controls and the tumour-bearing rats treated with fluoro-oleic acid ; there were no changes comparable with the fluorocitrate treated animals.

Effect of 1-*deoxy*-1-*fluoroglycerol treatment on rats with transplantable hepatoma.*— Twelve hepatoma-bearing rats were given 6 intraperitoneal injections of fluoroglycerol on alternate days (total 45 mg./kg.). They were killed 24 hr. after the last dose. The animals showed some malaise 3 hr. after the injections.

The total weight of tumour in these 12 rats was 195 g., slightly less than the total weight of tumour in 12 saline treated rats, 227 g. The difference is not significant. Five out of 12 rats treated with 1-deoxy-1-fluoroglycerol showed some lipid in the convoluted tubules compared with 0/12 in the control rats. The findings suggest that the fluoroglycerol might have some effect on the kidney, but that this was slight when compared with the effect of fluorocitrate.

DISCUSSION

Fluorocitrate produces a specific block in the citric acid cycle at the aconitase stage and thus causes an altered cell metabolism which is accompanied by an accumulation of citrate. The finding reported here that acute fluorocitrate poisoning in rats causes acute mitochondrial swelling in the cells of the proximal convoluted tubules with blockage of the tubules and distension of Bowman's capsule with fluid is therefore the pathological result of a specific biochemical lesion due to inhibition or blockage of one enzyme. In rats subjected to chronic poisoning, and killed or dying after 2 or more daily or alternate day doses, marked fatty change was observed in the proximal convoluted tubules, together with degeneration and loss of tubular cells. The rats dying in this group showed fatty degeneration in the walls of arcuate, interlobular and glomerular arteries. Thus the histological picture resembles that found in the kidneys of patients dying with lipoid nephrosis.

Human nephrosis is a syndrome which has many different causes (Squire, Blainey and Hardwicke, 1957). MacNider (1924) reviewed the literature on experimental nephrosis and Ehrich, Forman and Seifer (1952) discussed human and experimental nephrosis. Of the many specific agents which have been suggested as the cause of human nephrosis good evidence implicates poisoning by salts of the heavy metals, Cr, Ur, Au, Pb and especially Hg (Wilson, Thomson and Holzel, 1952) and mercurial diuretics. *Plasmodium malariae* has a possible aetological role (Gilles and Hendrickse, 1960). Carbon tetrachloride has been mentioned (Cataldi, 1958), and even a case following snake-bite has been reported (Steinbeck, 1960).

More light can be thrown on the problem by a consideration of the different aetiological agents which will produce a nephrosis-like syndrome in experimental animals. These agents include chromates, uranyl nitrate, mercury salts, cadmium salts plus 2,3 dimercaptopropanol (BAL), organic compounds of arsenic, Safranin O, tartrates, oxalates, deficiency of dietary potassium, large doses of cortisone or ACTH, specific anti-kidney serum, 6 aminopurine nucleoside, sodium tetrathionate,

alloxan, serotonin and finally fluorocitrate. Let us consider the mode of action of each of these agents to see if any pattern emerges.

Heavy metal poisoning.—Suzuki was quoted by Oliver (1915) as dividing the proximal convoluted tubules into three zones, proximal, middle and distal on the basis of a vital staining reaction with carmine—the most proximal part staining most heavily. Chromium salts damage the most proximal part and the uranium salts, mercuric chloride and cantharidin the most distal part (but cantharidin also causes marked damage to the glomeruli and is therefore ruled out of the discussion). Organic compounds of arsenic, especially arsacetin, arsenophenylglycine and atoxyl all give pale kidneys due to tubular damage (MacNider, 1924). (Arsenic itself and the other arsenical compounds cause extensive damage to the glomeruli as well as to the tubules.)

Peters, Stocken and Thompson (1945) found that the toxic action of mono-substituted tervalent arsenical compounds such as Lewisite (2-chlorovinyl dichloroarsine) or mapharside was due to a combination of the arsenical with two vicinal —SH groups to form a rather stable ring compound. The primary site of attack was some constituent of the pyruvate oxidase system, the action of which was inhibited ; BAL (2,3 dimercaptopropanol) reversed this inhibition. The action of disubstituted tervalent arsenicals, such as diphenylchlorarsine, was different, as this can attack a single —SH group in an enzyme (Peters, 1955). It was found that BAL was also a powerful antidote for mercuric poisoning in patients (Longcope and Luetscher, 1946), which was also studied in animals by Gilman, Allen, Philips and St. John (1946). In strong support of this observation is the finding of Wilson *et al.* (1952) that treating mercury nephrosis in young children with dimercaprol (BAL) not only cured the patients but also removed the body-burden of mercury via the urine. Thus the combination of the heavy metal with —SH groups in the renal cells is reversible by means of a thiol reagent. Concerning mercurial diuretics, Borghgraef and Pitts (1956) gave Chlormerodrin labelled with ^{203}Hg to rats and found that 80 per cent of the mercury localized in the kidney, largely in the cortex ; BAL reduced the amount of mercury in the kidneys and increased that of plasma, muscle and liver. Greif, Sullivan, Jacobs and Pitts (1956) found that the highest concentration of ^{203}Hg/mg. nitrogen was present in the "soluble" fraction of the cells, but bound mercury was also present in the "granular" fraction of the cells. Kleinzeller and Cort (1957) suggested that the mercury became firmly bound to the –SH proteins of the cell membrane thus increasing the permeability of the cells to Na, K, and protein and that this was the basis of the diuretic action. (It is, however, well recognized that leakage of cell K and gain of Na follows cell damage and in particular inhibition of mitochondrial ATP synthesis). Uranyl nitrate (1 mM) completely inhibits succinoxidase, hexose monophosphate oxidase and lysozyme, and partly inhibits cytochrome oxidase, amine oxidase, urease and pancreatic esterase. Meyer, Muntz, Singer and Barron (1951) did not consider that its action was by —SH inhibition.

Sodium tetrathionate and diffusible thiol-compounds.—Gilman, Philips, Koelle, Allen and St. John (1946) found that sodium tetrathionate injected into anaesthetized, catheterized dogs caused such a severe nephrotoxic action that there was complete anuria within 30–60 min. with necrosis and nuclear degeneration of cells in the proximal convoluted tubules. They found that tetrathionate oxidized soluble thiol compounds *in vitro* and the nephrotoxic action *in vivo* was abolished by prophylactic injections of 1-thiosorbitol or cysteine. Two alternative theories

have been suggested: (1)—that tetrathionate selectively penetrates the cells of the proximal tubule to exert a direct toxic action on enzymic systems dependent on thiol groups, or (2)—the cells of the proximal tubules are selectively damaged by the removal of diffusible thiol compounds (glutathione). Goffart and Fischer (1947, 1948) found that the protein bound —SH of the proximal tubules was not reduced in tetrathionate poisoning as judged by histochemical staining methods, but the reduced glutathione was greatly lowered on chemical analysis. They therefore favoured the second hypothesis. Unfortunately we cannot find in the literature any photomicrographs of these most interesting changes in the renal tubules, but the description sounds very similar to our findings with fluorocitrate.

Alloxan has a specific cytotoxic effect on the β cells in the islets of the pancreas. In addition a nephrotoxic action on the convoluted tubules has been frequently reported (Duff, 1945). Alloxan combines with thiol groups of protein and inhibits activity of succinic dehydrogenase (Lazarow, 1946). Its toxic action on the islet cells can be prevented by cysteine or GSH injected 1 min. before the alloxan. Alloxan also causes a rapid fall in the level of liver GSH to 17 per cent, and blood GSH falls to near zero. The mechanism by which the other diabetogenic drugs cause renal damage is not clear. Synthalin A (decamethylene diguanidine) produces a variable but usually severe degree of necrosis of the proximal convoluted tubules, there is however no fall of blood GSH during 1 hr. after injection (Davis, 1952). Another hypoglycaemic biguanidine, Phenformin, produces hyaline droplets in the distal convoluted tubules leading to degeneration of renal parenchyma and fibrosis (Lazarus, Bradshaw and Volk, 1960).

Safranin O.—Ginzler (1946) found that intravenous injection of Safranin O, 20 mg./kg., into rabbits or dogs produced selective renal tubular necrosis involving predominantly the proximal convoluted tubules. The histological picture resembled that in heavy metal poisoning: 40 mg./kg. caused almost complete necrosis of the proximal convoluted tubules as well as some damage to the distal tubules. There was a mild degree of fatty infiltration of the liver. Acriflavine and 2 (*p*-acetyl aminostyryl) 6 d-methylaminoquinolinemethochloride gave closely similar lesions.

Potassium deficiency and stress.—Potassium deficiency in rats caused diffuse tubular nephritis with the epithelium of the tubules markedly oedematous, vacuolated or sloughing in places. Ascites was present and there were many changes in the viscera including haemorrhage into the myocardium (Schrader, Prickett and Salmon, 1937). Kornberg and Endicott (1946) reported tubular casts and irregular dilatation of the collecting and convoluted tubules, but no fatty degeneration was seen. Milne, Muehrcke and Heard (1957) found the cells of the collecting tubules enlarged after 18 days of potassium depletion, by numerous eosinophilic hyaline granules 0·5 to 2μ in diam. MacPherson and Pearse (1957) studied the histochemistry of the kidney in potassium depletion. They found no change in the alkaline phosphatase, succinic dehydrogenase or 5 nucleotidase. After 8 days esterase increased and after 18 days the phosphorylase and TPN diphorase increased in the medulla and the DPN diphorase decreased. Potassium deficiency may act specifically on the kidney but it might act via the adrenal by a stress reaction. It is interesting to find that large doses of cortisone or ACTH induced renal changes in rabbits causing gross dilatation of tubules with enlargement of the glomeruli. Lipid was present in the tubular cells and casts were seen (Baxter, 1960).

6-*amino nucleoside*. Frenk, Antonowicz, Craig and Metcoff (1955) produced nephrosis in young rats by subcutaneous injections of 6 dimethylamino 9 (3′-amino β D-ribofuranosyl) purine. They found thickening of the glomerular basement membrane and marked loss of mitochondria with fatty degeneration of the infra-nuclear portions of the cells of the convoluted tubules. There were a few protein casts. There was much loss of albumin leading to ascites and subcutaneous oedema. Hoch-Ligeti (1960) found succinic dehydrogenase activity significantly reduced in the kidneys of the treated rats.

Specific anti-kidney serum.—Lindeman (1900) injected guinea-pigs with rabbit kidney and produced a nephrotoxic serum which on injection into rabbits induced marked albuminuria, formation of casts and tubular degeneration. Pearce (1904) perfused the kidney prior to using it as antigen to avoid production of haemolysins, and Smadel (1936, 1937) made many experiments to test the specificity. Histologically, during the acute phase after injection, there was extensive tubular degeneration, principally of the hyaline-droplet type, but dilated tubules lined by atrophic epithelium and filled with granular or protein casts were frequently seen. Fatty degeneration was not observed during the first week but was usually present in the tubular cells during the second week. Heymann and Lund (1948) and Ehrich *et al.* (1952) also produced a nephrosis-like condition by nephrotoxic sera. It would appear that the more specific the sera, the less the glomerular damage and the more the lesions were confined to the tubular epithelium.

Tartrates and oxalates produce acute tubular necrosis (MacNider, 1924), presumably by reducing the availability of Ca^{++} and thus interfering with essential tissue enzymes. Oxalate for instance interferes with lactate and malate dehydrogenase.

Fluorocitrate.—It is noteworthy how similar are the lesions we find to those produced by nephrotoxic sera and heavy metal poisoning. The primary biochemical lesion inflicted by fluorocitrate is a specific block of the citric acid cycle at the aconitase stage. There will, however, be a secondary accumulation of citrate and possibly this might be sufficient to immobilize calcium and act like tartrate or oxalate. Fluorocitrate is formed by a lethal synthesis from fluoroacetate in the S. African plant *Dichapetalum cymosum* and also from the fluoro-oleic acid in the seeds of the W. African *Dichapetalum toxicarium*. The latter is believed to have been used by " local doctors " for nefarious purposes. It is therefore possible that actual cases of poisoning by fluorocitrate may occur in man.

CONCLUSIONS

We see that nephrosis-like conditions appear after (1) heavy metal poisoning which inhibits —SH or other enzymes, (2) after tetrathionate or alloxan poisoning which reduces soluble —SH in the cell, (3) after potassium deficiency, (4) cortisone excess, (5) 6 amino nucleoside poisoning, (6) calcium active agents, (7) nephrotoxic sera and (8) blockage of the citric acid cycle by fluorocitrate. All these different agents would act as mitochondrial poisons and we would like to suggest that this is the common denominator of them all. For instance Hoch-Ligeti (1960) finds succinic dehydrogenase significantly reduced in nucleoside nephrosis and Philips, Gilman, Koelle and Allen (1947) find succinic dehydrogenase is powerfully inhibited by tetrathionate, as it is by the heavy metals. Histologically the hyaline droplet degeneration, the loss of mitochondria and the fatty change are all suggestive of mitochondrial damage. Tribe, Harvey and

Barcroft (1916) and Tribe, Hopkins and Barcroft (1916) found lowered oxygen consumption of intact kidneys *in vivo* rendered nephritic by uranium acetate or by diphtheria toxin. In the first case the renal blood flow was increased, in the second diminished. It is of course possible to get similar lesions by anoxic damage or by marked reduction of renal blood flow (Sheehan and Davis, 1960) and this is probably the mechanism by which large doses of serotonin causes tubular necrosis and even renal cortical necrosis (Waugh and Pearl, 1960).

We must still find an adequate explanation for the way in which different poisons attack different parts of the tubule, if we are to reconcile fact and theory. Chromium salts and fluorocitrate damage the first part of the proximal convoluted tubule, uranium salts damage the distal part of the proximal convoluted tubule. We believe there is a simple reason for this. The diffusible fluorocitrate and chromium salt would be present in the glomerular filtrate and would be concentrated in the first part of the proximal convoluted tubule. The heavy metal might be more tightly bound to protein and would reach the tubule via the efferent arteriole which breaks up into capillaries over the ascending loop of Henle and distal part of the proximal convoluted tubules (Trueta, Barclay, Daniel, Franklin and Pritchard, 1947). If this hypothesis is correct then tartrates, oxalates, tetrathionate and alloxan should all give a picture like fluorocitrate, but unfortunately we can find no data on this point. Cadmium is not normally nephrotoxic but becomes so if given with BAL which renders it soluble and so capable of redistribution (Ginzler, Gilman, Philips, Allen and Koelle, 1946).

Glomerular damage has tended to monopolize attention because of the dire results which follow in its wake. There has, therefore, always been the desire to explain the protein loss which occurs in nephrosis by changes in the glomeruli. Vernier, Papermaster and Good (1959), Harkin and Recant (1960) have made electron-microscope investigations of the renal damage in 6 amino nucleoside nephrosis. They find alterations of the glomerular epithelium with fusion of the foot processes and cytoplasmic vacuolation. But the earliest lesions are focal swellings between the cristae of the mitochondria of the glomerular epithelium. A very apt reminder that the epithelial cells of the glomeruli have mitochondria too, and that mitochondrial poisons may be able to damage the glomeruli as well as the tubules. In fact we should expect this to occur, to a greater or lesser extent, with all the agents we have discussed here, and this is precisely what is found. The fact that glomerular damage may be progressive and permanent, while tubular damage may be reparable, should not blind us to the importance of tubular damage, or to the fundamental nature of the underlying biochemical-pathological lesion.

SUMMARY

The kidneys of rats dying 2 hr. after 60 or 80 mg./kg. fluorocitrate i.p. showed degeneration of the mitochondria in the cells of the proximal convoluted tubules with blockage of the tubules and distension of Bowman's capsule with fluid.

In rats killed, or dying, after 2 or more daily or alternate-day doses, marked fatty change was observed in the cells of the proximal convoluted tubules, together with degeneration and loss of tubular cells. Rats dying in this group showed fatty degeneration in the walls of the arcuate, interlobular and glomerular arteries. The histological picture resembled that found in the kidneys of patients dying with lipoid nephrosis. Similar changes were found in hepatoma-bearing rats treated

with fluorocitrate, but not in the kidneys of hepatoma-bearing rats injected with saline, fluoro-oleic acid, or 1-deoxy-1-fluoroglycerol.

Fluorocitrate produces a specific block in the citric acid cycle at the aconitase stage. The various known causes of nephrosis in rats are discussed in relation to their action as mitochondrial poisons.

We wish to express our thanks to Dr. D. H. Treble and Miss P. Ivall for help with some of the preliminary experiments, to Mr. S. Davison for making the estimations of serum protein and cholesterol and to Miss N. C. Burr for technical assistance. One of us, R. A. P., wishes to thank the Wellcome Trustees for a grant towards expenses.

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EXPERIMENTAL NON-DIETARY CIRRHOSIS IN RATS

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EXPERIMENTAL cirrhosis is at present most conveniently studied in rats, either by depriving the animals of lipotropes, sulphur-containing amino acids or α-tocopherol, or by the repeated administration of hepatotoxins. This paper describes an apparently unrelated method in which cirrhosis follows the intravenous injection of egg yolk. The pattern of fibrous tissue production after egg yolk is similar to that described in diffuse hepatic fibrosis (Himsworth, 1947), but egg yolk cirrhosis differs from diffuse hepatic fibrosis in not showing fatty change at any stage. The cirrhosis after egg yolk superficially resembles an early or mild Laennec type cirrhosis, but unlike the human disease it is not progressive and features of portal hypertension such as ascites fail to appear. Objection might be raised to the use of the term cirrhosis in this connection, but it has been used purely for convenience throughout this paper. Egg yolk cirrhosis was first observed when egg yolk injections were combined with unilateral renal pedicle ligation, and the results then obtained are included in this paper, though subsequent work has shown that renal pedicle ligation is not essential for the production of the cirrhosis.

MATERIAL AND METHODS

Thirty-four male albino rats weighing initially between 150–300 g. were used in this experiment and were fed rat cubes and water *ad libitum*.

Fresh hens' eggs provided the egg yolks and these were separated from the whites without special aids so that small amounts of egg white were almost certainly included in the yolk, which was then diluted 1 : 1½ with isotonic saline, filtered through gauze, tested for sterility and given in doses of 2 ml./100 g. body-weight under ether anaesthesia to all groups of rats.

In the first experiment 17 rats were given an intravenous injection of egg yolk on the first day, the left or right renal pedicle tied 4–6 hours later and intravenous egg yolk injections continued every 2nd–4th day for 19–88 days. Postmortem examinations were done on 15 of these 17 rats, preceded by liver biopsy in 2. The remaining 2 animals in this group are still alive and in them proof of cirrhosis is based on liver biopsy alone.

In a second group of 17 rats, renal pedicle ligation was omitted and egg yolk injections alone given every 2nd–4th day for 18–145 days. Liver biopsies only were examined in 12 of these animals and the other 5 were subjected to postmortem examination without prior liver biopsy.

Tissues were fixed in formol-saline, embedded in paraffin wax and stained with haematoxylin and eosin. The Prussian blue reaction was used to demonstrate iron, the Van Gieson method for collagen and very dilute aqueous solutions of toluidine blue for metachromasia.

RESULTS

Intravenous injection of egg yolk, even in the relatively enormous amounts used, produced neither shock nor respiratory distress, and though some animals appeared inactive and listless for about 30 min. after injection, they remained

warm and were easily roused to activity. A regular sequel to the first injection of egg yolk, however, was the development within 10–15 min. of oedema of the paws, nose scratching and redness of the ears, the latter lasting some minutes but the paw oedema persisting sometimes for 4–5 hr. These signs were occasionally seen after the second egg yolk injection but not after further injections.

A definite cirrhosis or changes suggesting early cirrhosis were present in all 17 rats given egg yolk after renal pedicle ligation, and similar lesions occurred in all but 2 of the 17 given egg yolk injections alone. In no animal was cirrhosis accompanied by even mild fatty change, and no necrosis of massive, zonal or focal type observed, though isolated dead liver cells were detected microscopically. The lesions caused by egg yolk alone are so similar to those which follow egg yolk combined with renal pedicle ligation, that no distinction is made between the two groups in the descriptions which follow.

At autopsy the liver was pale brown in colour, cirrhotic to the naked eye with a finely and uniformly granular external surface. The weight of the liver appeared to increase as the experiment progressed. The lowest weight was 9 g. and this was recorded in 2 rats of 230 and 203 g. killed after 28 and 30 days, while the highest values of 16 and $15\frac{3}{4}$ g. were found in 2 animals weighing 285 and 300 g. and killed after 47 and 170 days. In 15 animals of the experimental group, weighing 203–300 g., the average weight of the liver was 12·2 g. with a range of 9–16 g., and in 12 normal rats weighing 240–422 g. the average weight of the liver was $11\frac{1}{2}$ g. and the range 8–$16\frac{1}{2}$ g.

The earliest histological lesions were seen in 4 rats killed 18–21 days after the start of the experiment when each had received 7 egg yolk injections (Fig. 1). Hepatic lobular architecture was still recognisable in these cases, but a heavy infiltration of eosinophils, plasma cells, mast cells and iron-laden macrophages was seen in portal tracts and around hepatic veins, while a few fibroblasts from these areas invaded the lobules in relation to small groups of necrotic liver cells. The liver cell necrosis was an inconspicuous and piecemeal process affecting single cells or small groups of three or four cells along the periphery of the lobule. The affected cells shows loss of central nuclear chromatin and its condensation along the nuclear membrane in dense round masses. Pyknosis followed, with one or two small dark nuclear masses in a homogeneous deeply eosinophilic cytoplasm. Single necrotic cells, which were often large and retracted away from their neighbours, apparently evoked little or no reaction, but each small group of necrotic cells near a portal tract or hepatic vein was accompanied by a vascular, fibroblastic and cellular response, the last being largely of eosinophils. The sinusoids in the region of the necrotic cells were dilated and their lining cells plump and numerous so that they resembled capillary channels. The liver cells and their nuclei in these 4 animals appeared large when compared with sections from normal animals, but more definite evidence of regeneration was provided by the increased mitotic activity that was seen in each case, with an average of 3 mitotic figures per low power field.

The early hepatic lesion just described quickly progressed to a cirrhosis of monolobular type. This was seen as early as 21 days after the first of 9 egg yolk injections in 1 animal and was present in 5 others killed after 28–30 days when 8–14 injections had been given. Thin bands of fibroblastic or collagenous tissue surrounded or intersected the lobules as in a pig's liver, but this resemblance was fallacious, as fibrous bands often passed between hepatic veins or joined

hepatic veins to portal tracts. This established Laennec type cirrhosis seemed however to be still active : the infiltration of eosinophils, plasma cells, mast cells and iron-laden histiocytes persisted in portal tracts, isolated necrotic liver cells could still be found at the periphery of the lobule, while increased mitotic activity was still pronounced and apparently much in excess of what one would have expected as a replacement for the isolated necrotic liver cells. Bile duct regeneration provided further evidence of an active lesion, and increased numbers of small ducts, apparently recently formed, were recorded in 5 of the 6 animals in this group.

In all the remaining 22 animals which were killed or biopsied for the first time 34–170 days after starting the experiment, an established monolobular Laennec type cirrhosis was present (Fig. 2) with thin fibrous bands surrounding irregularly shaped or rounded regeneration nodules which were often much smaller than a normal liver lobule. But despite the continued egg yolk injections, no fresh additions were made to the fibrous tissue bands and these remained uniformly thin. The eosinophil infiltration, the isolated necrotic liver cells (Fig. 3) and the excessive mitotic activity in the healthy liver cells could still be seen, but even these features appeared gradually to diminish and finally disappeared. Thus, in 4 biopsies taken on the 70–88th days, mitoses and necrotic liver cells were not seen and infiltrating eosinophils difficult to find, while in one animal biopsied for the first time after 130 days, cirrhosis was unaccompanied by any such signs of activity (Fig. 4). In this animal the picture was simply that of so-called reversed lobulation, with relatively normal sized pentagonal lobules bounded by regular, straight fibrous bands. There is a vast difference between these histological appearances and those illustrated in Fig. 2, but this is of course not proof that all the earlier lesions regress to the quiescent lesion seen in Fig. 4. The comparison here is between different animals examined for the first time at different stages of the process and is made on autopsy material on the one hand (Fig. 2) and the rather more restricted biopsy material on the other (Fig. 4). Nevertheless, if the cirrhosis did not actually regress in this animal (Fig. 4), it certainly did not become severe despite continued egg yolk injections. Nor was the cirrhosis progressive when egg yolk injections were stopped for 17–24 days in 6 rats and for 85 days in one animal, as judged by serial biopsies.

From the whole sequence of morphological changes, it appears that an intense response to egg yolk injections occurs 2–3 weeks after the start of the experiment and leads quickly to a mild Laennec type portal fibrosis. After this initial active period the lesion does not progress, even when egg yolk injections are continued.

In none of the animals killed at any stage was there evidence of tubular necrosis in the kidney, except of course in the group where one renal pedicle

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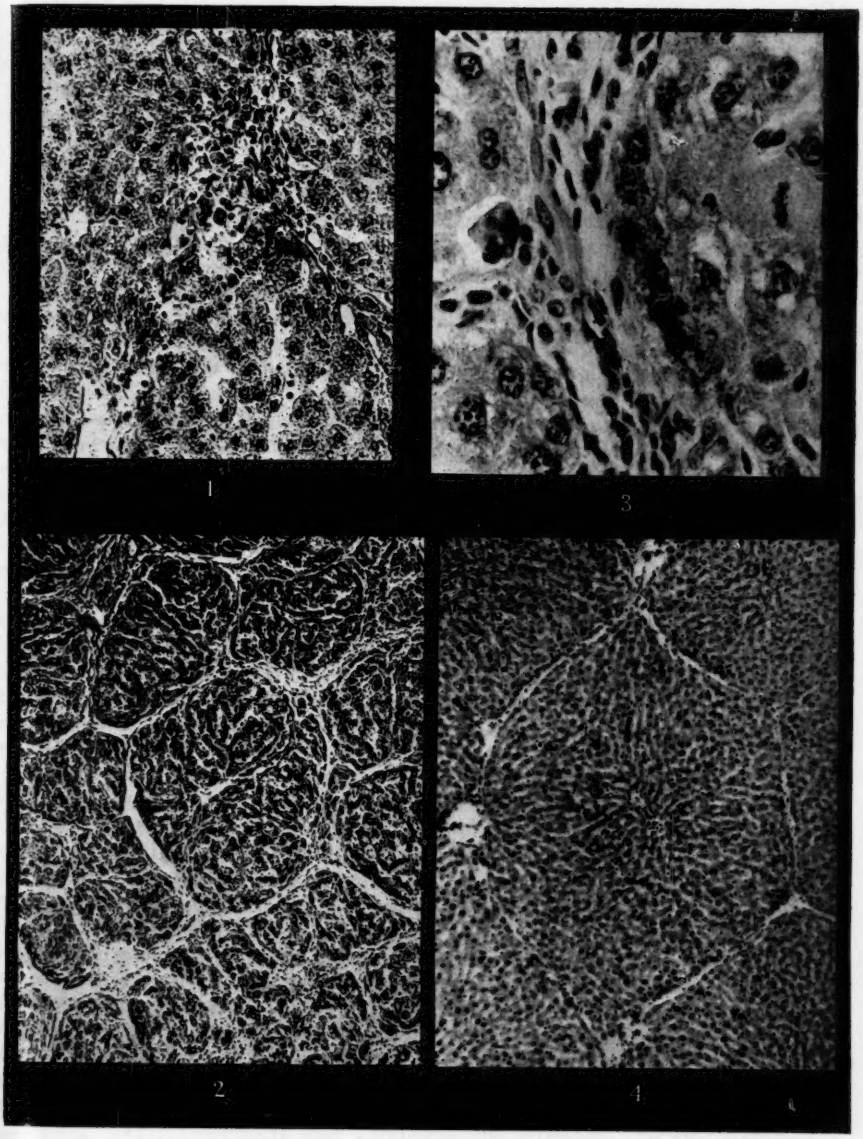
EXPLANATION OF PLATE

FIG. 1.—The earliest histological lesion 18 days after the first of 7 egg yolk injections. (H. and E. ×190.)

FIG. 2.—An established monolobular cirrhosis 47 days after left renal pedicle ligation and 14 egg yolk injections. (H. and E. ×76.)

FIG. 3.—An isolated necrotic liver cell in the upper half of the field and a mitotic figure in the lower half. This animal was given 12 egg yolk injections in 34 days following left renal pedicle ligation. (H. and E. ×495.)

FIG. 4.— So-called reversed lobulation in the first biopsy taken 130 days after 45 egg yolk injections. (H. and E. ×76.)



Campbell.

was tied. The splenic pulp in most cases appeared to contain more plasma cells than in control animals, while the lungs, apart from the occurrence of senile bronchiectasis, showed occasionally a foreign-body giant cell response, possibly due to particulate matter in the egg yolk. Brain, myocardium, aorta, adrenal glands and testes showed no noteworthy lesions.

DISCUSSION

The experiments show that a non-progressive cirrhosis may be easily and rapidly produced in rats by the simple expedient of egg yolk injection. There is no evidence that renal pedicle ligation will alter the final results.

Most types of experimental cirrhosis differ in varying degrees from human cirrhosis and egg yolk cirrhosis is no exception. It differs strongly however from other types of experimental cirrhosis in the following respects: the swiftness with which cirrhosis develops (21–28 days), the absence of even mild hepatic fatty change, the lack of extensive necrosis in the liver and the absence of a renal tubular lesion. The antecedent necrosis or prolonged severe fatty change which occur in other types of experimental cirrhosis are widely accepted as the cause of the fibrosis. In egg yolk cirrhosis, antecedent hepatic lesions and the time factor are so unexpectedly different from those customarily described that it seems difficult to apply the usual explanations of direct hepatotoxic action, or of deficiency of lipotropes, vitamin E or sulphur-containing amino acids to the results with egg yolk. If, for example, egg yolk exerts hepatotoxic effects by its content of foreign protein, peptones or amino acids, then these effects must be cumulative and delayed, for acute liver damage does not follow a single injection but is only seen after several very large injections have been given. It follows too that the liver must lose its original capacity to react with egg yolk as injections given after cirrhosis has developed cause no further damage in the liver. On the same basis a deficiency of any essential food factor would seem unlikely in these animals.

The paw oedema and other signs which follow the first intravenous injection of egg yolk suggest the possibility of histamine liberation in these experiments. Paw oedema, redness of the ears, nose scratching and prostration follow injections of egg white in the rat and are due to release of histamine (Feldberg and Talesnik, 1953) and 5-HT (Rowley and Benditt, 1956). It is similarly possible that egg yolk releases histamine, that this then attracts eosinophils to the liver (Archer, 1959) and produces the vascular response there. The hypothesis that vascular disturbance within the liver in chronic histamine intoxication might be important in the pathogenesis of cirrhosis is not a new one. It was proposed by Eppinger and Leuchtenberger (1932) but the lesions they illustrated appear to be those of hepatic atrophy and are different from those which follow egg yolk injection. More recently Ogata (1956) produced a "congestive cirrhosis" in rabbits by partial ligation of the inferior vena cava, and reports from his laboratory show that many substances including histamine (Sakamoto, 1958) and particularly egg white (Akiyama, 1957) accentuate and accelerate the cirrhosis. In the experiments with egg yolk a relationship to histamine intoxication is less obvious, for the paw oedema and other signs taken as evidence of histamine release, are seen only at the beginning of an experiment that lasts for several weeks. It is possible that rats are desensitised to egg yolk during this time, and that release of hist-

amine, or 5-HT, has little to do with the subsequent cirrhosis. Two points appear to offer some confirmation of this. The first is the statement by Feldberg and Talesnik (1953) that 10–14 days are required for regeneration of histamine after its release : the 2–3 day intervals between egg yolk injections would seem quite inadequate for this to occur. The second is the failure to produce any feature of cirrhosis in a separate group of 8 rats given repeated intraperitoneal injections of the histamine liberator 48/80. On the other hand, the intravenously injected egg yolk is in intimate contact with the Kupffer cells of the liver and such intimate contact may lead to different local results. It is also true that 48/80 cannot deplete the stomach and liver of histamine as completely as it can the skin and muscles (Feldberg and Talesnik, 1953).

Egg yolk cirrhosis has however an even older experimental precedent in the work of Wells (1920). A footnote in the fourth edition of his ' Chemical Pathology ' reads as follows : " In a paper appearing in the Transactions of the Chicago Pathological Society, 1903, **5**, 240, I published the observation that repeated injections of Witte's ' peptone ' (which consists chiefly of proteoses) into rabbits led to the production of marked cirrhosis of the liver, and suggested the possibility that proteoses escaping through a diseased gastric or intestinal wall into the blood might be a factor in the production of cirrhosis in man. Subsequent observations, however, have shown that repeated injection of almost any foreign protein material (*e.g.*, emulsions of organs, foreign blood, etc., used in immunization experiments) will cause similar cirrhosis in rabbits, which animals, indeed, often spontaneously show this condition when apparently otherwise normal. ' Peptone ' injections in dogs and guinea-pigs have failed to cause a similar cirrhosis, and hence the value of these and all other rabbit experiments on cirrhosis of the liver is very questionable ; however, the possibility of the correctness of the original conclusions still remains open." The similarities between Wells's observations and my own are very great, particularly as I too have found that other protein materials such as egg white, chick embryo extract, horse serum and sterilized milk produce a similar cirrhosis in the rat in a comparable time period. The common factor is the introduction of foreign protein and though this is far removed from current ideas on the pathogenesis of human cirrhosis, Longcope in 1913 (quoted by Moon, 1934) recorded hepatic necrosis and subsequent perilobular fibrosis after repeated anaphylactic intoxication. The antigens used were egg white and serum albumin and the experiments were successful in cats, dogs, rabbits and guinea-pigs. Two points in my own experiments suggest that an allergic response to egg protein might occur at some stage. The first is the predominance of eosinophils in the hepatic cellular response, and the second the fact that the 14–21 days which elapse before one finds microscopical evidence of response to egg yolk is also roughly the period required for the development of antibodies.

Although one cannot say what component of egg yolk causes the cirrhosis or how it produces it, it is clear that a vascular and cellular response, accompanied by only trifling and molecular liver cell necrosis occurs when egg yolk has been given to rats for 2–3 weeks, and that death of isolated liver cells antedates cirrhosis by some 7–10 days. The fibrosis occurs roughly in the regions where one previously observed isolated necrotic liver cells, but it is not at all clear whether the fibrosis is the result of the necrosis. A causal relationship between liver cell necrosis and hepatic fibrosis is however firmly established in experimental path-

ology, and Cameron and Karunaratne (1936) in discussing their work with carbon tetrachloride, suggest that a factor released from autolysing necrotic liver tissue stimulates both stroma and parenchyma to activity. Hepatic necrosis in the present experiments is so insignificant that such an explanation seems difficult to apply, particularly when one considers the short, relatively constant period in which fibrosis develops, the intensity of regeneration in the face of trifling necrosis, and the failure of the fibrosis to progress or necrosis to persist in spite of regularly continued egg yolk injections.

SUMMARY

Repeated intravenous egg yolk injections produce a fine monolobular cirrhosis in rats in 3–5 weeks. Fibrosis is preceded by a brisk cellular infiltration and a degree of regenerative activity out of all proportion to the trifling amount of liver cell necrosis that occurs, but hepatic fatty change is quite absent. Fibrous tissue formation is however not progressive, ascites uniformly fails to develop and all hepatic activity appears to cease within 130 days of starting the experiment.

The changes do not seem to be explained on the basis of direct hepatotoxic action or deficiency of dietary factors, and although histamine and 5–HT are probably released in the early stages of the experiment, histamine at least does not seem to be directly responsible for the fibrosis. The introduction of foreign protein, which forms the basis of these experiments, may however be a factor in the pathogenesis of this experimental lesion.

Professor J. G. Thomson offered much helpful criticism and kindly read the manuscript. Mr. W. Taylor and his staff prepared the sections, while the 48/80 used in these experiments was a gift from Dr. White of Burroughs Wellcome and Co., England.

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SURVEY OF PAPERS

HUTTERER, FITSCHEN and POPPER have studied the accumulation of liver fat in relation to mitochondrial damage in rats during ethionine intoxication. Although these manifestations of injury can occur simultaneously, they appear to be aspects of the intoxication without causal relationship to one another (p. 187).

DAVIDSON, HOWARD and GRESHAM investigated the coagulation properties of the blood of rats. The control group were fed on 10 per cent arachis oil ; the thrombogenic group were given a diet containing 40 per cent butter, 20 per cent cholic acid, 5 per cent cholesterol and 0·3 per cent thiouracil, which induced thrombosis : the atherogenic group were fed on a similar diet but 40 per cent arachis oil was substituted for butter and this induced atherosclerosis. Blood samples were collected from these experimental animals and coagulation tests were performed.

It was noted that shortened coagulation times were found in the thrombogenic and atherogenic groups in the stypven time, the one-stage prothrombin time and two-stage plasma and serum prothrombin times, and the platelet counts were also elevated. The silicone clotting times were prolonged only in the thrombogenic group while the abnormal reduction in the serum prothrombin time and the platelet elevation were greater in the thrombogenic group (p. 195).

BAUER has determined the " zero effect dose " (E_0), the largest dose of compound which fails to affect the natural progress of the disease, in the reaction between isatin β-thiosemicarbazone and various pox viruses. He found for each particular pox virus that E_0 was constant regardless of virus dose. The viruses of the group could be placed in order of increasing E_0, *i.e.* increasing resistance. He points out that drug resistance as a virus marker character is not either present or absent, but varies in degree, that this may apply to other markers, and that, therefore, such characters cannot be dependent upon a single " gene " (p. 201).

KARNOVSKY has shown that 2-mercapto-ethylamine, a known osteolathyrogenic compound, has a direct glycogenolytic action on the cells of the epiphyseal cartilage of rats. In this respect it resembles amino-acetonitriles (p. 207).

MELLETT, WALTER and HOUGHTON exposed areas of skin (2·6 cm. diameter) on both flanks of albino guinea-pigs to X-rays (total dose 5000 r/animal). Test animals were given 10 mg. cortisone acetate daily for the next 42 days. Controls received no cortisone. They have described the gross and microscopic appearance of skin lesions in test and control animals, and have discussed their findings at length (p. 212).

WALKER and ROGERS found that in rabbits deprived of the greater omentum, the antibody response to i.p. injections of type III pneumococci was diminished.

Omentum from immune donors grafted in non-immune recipients subsequently injected with amino acids labelled with ^{14}C was shown by autoradiography of antigen-antibody precipitates to be producing antibody. Other possible origins of this radioactive antibody were excluded by studying the time of its appearance and by inoculating the donors with ^{131}I labelled anti-pneumococcal serum before the transfer of omentum, and then examining recipients' serum after grafting but before administration of ^{14}C (p. 222).

SMITH described the preparation and discusses the advantages of a tissue culture maintenance medium. This is a diluted ultrafiltrate of a peptic digest of liver (p. 232).

FLOREY, GREER, POOLE and WERTHESSEN investigated the structure of the pseudointima lining fabric grafts in the aortae of baboons. " Dacron " grafts were inserted in the lower abdominal aorta and the animals were killed approximately 10 weeks later. Histology confirmed that the inside of the graft was lined by a layer of tissue, the pseudointima. The graft was completely covered by endothelium and this was confirmed by the appearances of surface

preparations and in sections examined by electron microscopy. The pseudointima was also found to contain smooth muscle cells, polymorphonuclear leucocytes and foreign body giant cells. Fibrous tissue was present and this consisted of collagen, elastin and possibly reticulin. It was noted also that small blood vessels arose from the lumen of the graft and extended into the interstices of the fabric of the graft (p. 236).

DUCHEN investigated the effects of portal vein ligation on liver necrosis induced by injections of carbon tetrachloride into rats. The rats were divided into 4 groups, one had ligation of the portal vein branches to the anterior lobes ; in the second group this was followed by a subcutaneous injection of carbon tetrachloride ; the third group received carbon tetrachloride after ligation of only one branch of the portal vein and the fourth, or control group, had a laparotomy and injection of carbon tetrachloride only. The animals were subsequently killed and the amount of liver necrosis was assessed. This was performed by projecting 6 consecutive histological fields of the livers onto tracing paper. Areas of necrotic and healthy cells were outlined and the paper cut off. The papers representing these areas of the liver were then weighed separately and the necrosis expressed as a percentage of the total weight. It was found that the lack of portal blood did not increase the extent of the liver necrosis caused by carbon tetrachloride nor was the process of liver regeneration impaired (p. 247).

KATO and OKADA, by adsorption to chicken erythrocytes, separated the haemagglutinin from the soluble antigen of PR8 influenza A virus disintegrated by sonic oscillation. They found that virus toxicity in contrast to infectivity was enhanced by sonic oscillation, that most of this toxicity was found with the haemagglutinin, but that it was distinct from haemagglutinin in being destroyed by heat, UVL, and ethylmercurithiosalicylate. Some of the toxicity was found with the soluble antigen, and in this fraction toxicity and antigenic activity were sedimented together in the ultracentrifuge (p. 253).

RUTH JAMES and MACLEOD have studied the induction of staphylococcal infections in mice by introducing the organism on various suture materials. Local abscesses were produced by *Staph. pyogenes* introduced on various materials, but no differences in virulence were demonstrated between various strains of the organisms. Coagulase negative staphylococci did not give rise to abscess formation The potentiating effect of the suture materials appeared to be related to the severity of the inflammatory reaction produced by the sterile suture materials and to the power of these materials to pick up the organisms from broth culture (p. 266).

CATER and PETERS investigated the renal changes induced in rats following the intraperitoneal injection of fluorocitrate. Fluorocitrate produces a block in the citric acid cycle at the aconitase stage and causes altered cell metabolism accompanied by accumulation of citrate. The experimental rats used were poisoned with fluorocitrate which resulted in fatty changes in the proximal convoluted tubules and fatty degeneration of the arteries in the kidneys. The appearances were similar to those found in human cases of lipoid nephrosis and they discuss the various known causes of nephrosis in rats in relation to their action as mitochondrial poisons (p. 278).

CAMPBELL gave repeated intravenous injections of egg yolk diluted with isotonic saline in a dosage of 2 ml./100 g. bodyweight. These injections were given to 34 rats every 2nd–4th day for periods varying from 18–145 days. After the first injection oedema of the paws, nose-scratching and redness of the ears followed 10–15 min. later and the paw oedema persisted for up to 5 hr. The animals were either killed and the livers subjected to histology or liver biopsies were taken. All but 2 of the animals developed cirrhosis, this arose between the 3rd–5th weeks, but was not accompanied by fatty change and liver cell necrosis was negligible. The fibrous tissue formation did not appear to be progressive and ascites did not develop. He suggests that the introduction of foreign protein material was the main factor in the pathogenesis of this experimental cirrhosis (p. 290).